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The Analysis of Genetically Engineered Polyketide Metabolites

By

Dominic Kearney

Thesis submitted to the Open University for the Degree of
Master of Philosophy

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For Alice

Abstract

Experience has shown that genetically engineered, truncated polyketide synthases sometimes gave very low production levels (as low as 1-2mg per litre broth culture). To understand how the protein producing them works, it is important to be able to characterise the metabolites. To do this effectively these small quantities of product must be isolated from the broth so that NMR spectra may be obtained.

Within this thesis is described details of the initial analysis by GC-MS, the separation and subsequent isolation by preparative HPLC-MS and the interpretation of the NMR spectra. Work is also described relating to the pre-HPLC cleanup of biological extracts including flash silica chromatography and size exclusion chromatography. One of the compounds characterised has proven to have different stereochemistry to that expected based on previous knowledge. The structure, including stereochemistry of this new compound was obtained by a combination of a process of elimination (spiking studies) and by NMR spectra.

A comparative study of the effectiveness of HPLC-MS and GC-MS for the accurate quantification of triketide lactones has shown that GC-MS is the more preferable method having lower detection limits for this particular group of compounds.

The synthesis of a Δ -lactone is also described (this was used as a standard in both quantification and spiking studies).

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Chapter 1

Introduction

This chapter will briefly introduce the polyketide family of natural products and their biosynthesis, and then discuss the analytical techniques employed in the work described in this thesis, namely mass spectrometry, gas chromatography and high performance liquid chromatography.

1.1 Polyketides

Metabolism may be divided into two areas, primary metabolism and secondary metabolism. Primary metabolism usually refers to the biological synthesis of chemical compounds that play an essential role in the life of the organism producing them, for example sugars, lipids and of course DNA. Secondary metabolism mainly refers to the biological synthesis of compounds that may appear to have no noticeable benefit to the organism producing them. However, on closer inspection many secondary metabolites do show some function. Also, compounds such as pheromones may not be of direct use to the cell but may be of fundamental importance to the organism as a whole. These secondary metabolites are often created in times of stress or hardship.¹

The main area of concern in this thesis is with the group of natural products known as polyketides.

The name **polyketide** is used to describe a large group of naturally occurring secondary metabolites that are both structurally diverse and yet have a number of features in common.² The similarity of these polyketides derives from the simple building blocks that go into forming their sometimes quite complex structures. This comes about by the successive Claisen-type decarboxylative condensation of malonyl or substituted malonyl thioester derivatives with units derived from the coenzyme A thioester of carboxylic acids,³ leading to an iterative two-carbon chain extension. It is this way of building up the carbon skeleton which is used to classify polyketides.²

Complex polyketide biosynthesis occurs within multifunctional enzymes in a similar way to fatty acid synthesis. These enzymes are called polyketide synthases (PKS) and can have molecular weights in excess of 300,000.⁴

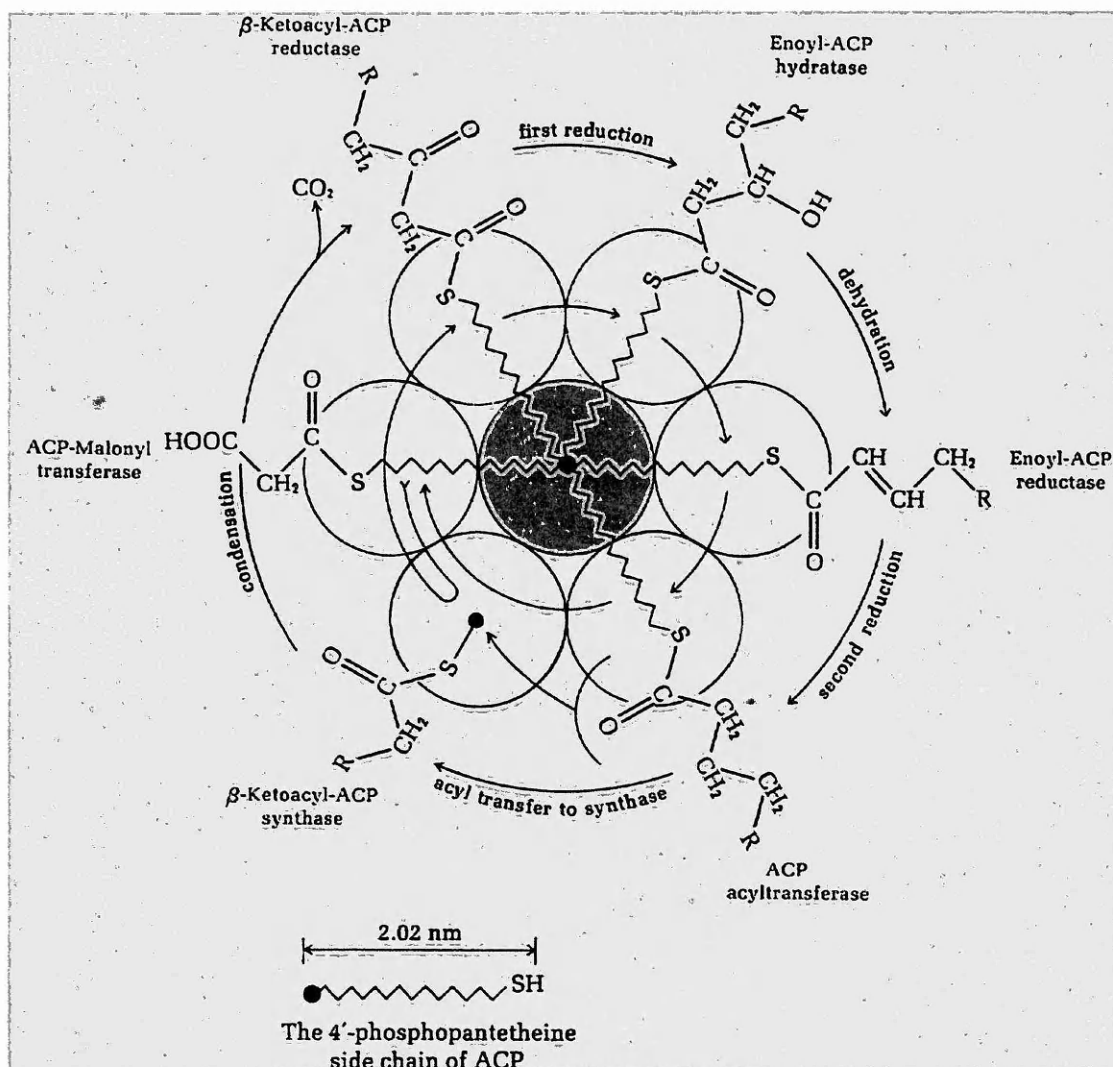
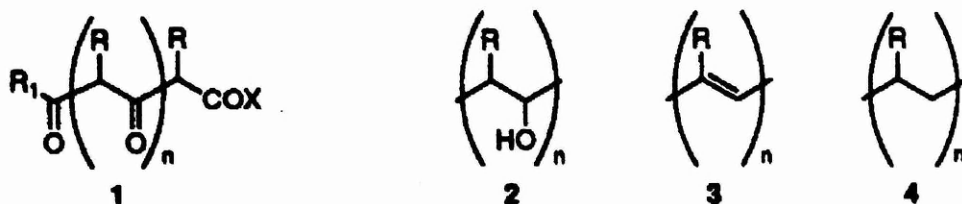


fig 1.1.1 Fatty-acid synthase.⁵ The central protein is ACP. The phosphopantetheine side chain serves to carry acyl groups from one enzyme area to the next

Polyketides may be divided into two main categories, the **aromatic** and the **aliphatic**. Aromatic polyketides are normally biosynthesised on type II, or iterative, synthases. Fatty acid biosynthesis (fig 1.1.1) occurs in much the same way as polyketide biosynthesis, in that they both proceed by a repeating iterative process around a single modul.⁵

However, unlike fatty acid synthesis, where the chain extender residue is fully reduced each cycle, aromatic polyketide synthases do not possess any reductive capabilities so the

resultant chain has a full keto group at each chain link β -position (1) (fig 1.1.2). This excess of keto groups along the chain facilitates aldol or Claisen type intramolecular condensations and hence the formation of an aromatic polyketide. Aliphatic polyketides, however, are often biosynthesised on type I, or modular synthases where the growing chain is passed along the enzyme from module to module, each one adding an extension residue. In these systems, each module can have varying levels of reductive capability. The structural diversity observed in aliphatic polyketides arises from the varying degree of reduction that each link may undergo at the β -carbon position (fig 1.1.2) to hydroxyl (2), alkenyl (3) or methylene groups (4), after it has been attached to the chain. Unlike fatty acid biosynthesis where the link is fully reduced after each condensation step, the modular polyketide synthase consists of discrete areas of enzymatic activity for every step of chain growth.



The Archetypal Poly- β -keto Chain Structural Variations Produced by Reduction of Keto Groups

fig 1.1.2⁶

These areas or modules are arranged along the enzyme in the order in which they are utilised. Within these modules is a series of smaller domains, which determine the structural nature of the resultant polyketide. These domains are analogous to ketosynthase (KS), acyltransferase (AT) and acyl carrier protein (ACP) on the fatty acid synthase (fig 1.1.1), all of which are essential to the chain extension cycle. Each module on the aliphatic polyketide synthase (fig 1.1.3) also contains a number of optional domains, the ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER), which are responsible for the modification of the newly formed keto group.⁷ The thioesterase (TE) domain is

responsible for catalysing the cyclisation reaction of the fully formed chain prior to its departing the enzyme. Each of these domains performs a different and very distinct role and it is the varying combination of these domains within each chain extending module which gives rise to the vast degree of structural diversity of polyketides not only in terms of the level of reduction but also stereochemistry.

An important example of is the **6-deoxyerythronolide B** synthase (fig 1.1.3) or DEBS produced by the bacterium *Saccharopolyspora erythraea*.⁸ This multifunctional enzyme complex is made up of three proteins⁹ known as DEBS1, DEBS2, and DEBS3. These enzymes are responsible for catalysing the biosynthesis of the macrolactone 6-deoxyerythronolide B^{10,11}, the skeleton for the antibiotic erythromycin A. The DEBS proteins, or cassettes, each incorporate two chain extension modules, each of which is responsible for adding one methylmalonyl-CoA chain extender unit (fig 1.1.4), giving six in total to form seven links in all (a heptaketide). In each of these modules are the domains necessary to build the macrolactone, or macrolide as it is more commonly known, in the required way. In the case of *S. erythraea*, there are certain post-PKS reactions that, among other things, add sugars to turn 6-deoxyerythronolide B into the antibiotic erythromycin A.¹² Erythromycin A belongs to a larger family of macrolides which is characterised by 12, 14 or 16-membered rings.² It was first isolated from *Saccharopolyspora erythraea* in 1952⁸ and is used as an antibiotic against Gram positive bacteria such as *Legionella pneumophila*, the bacterium which causes Legionnaire's disease.

Erythromycin PKS: Primary Organisation of the Genes and Proteins

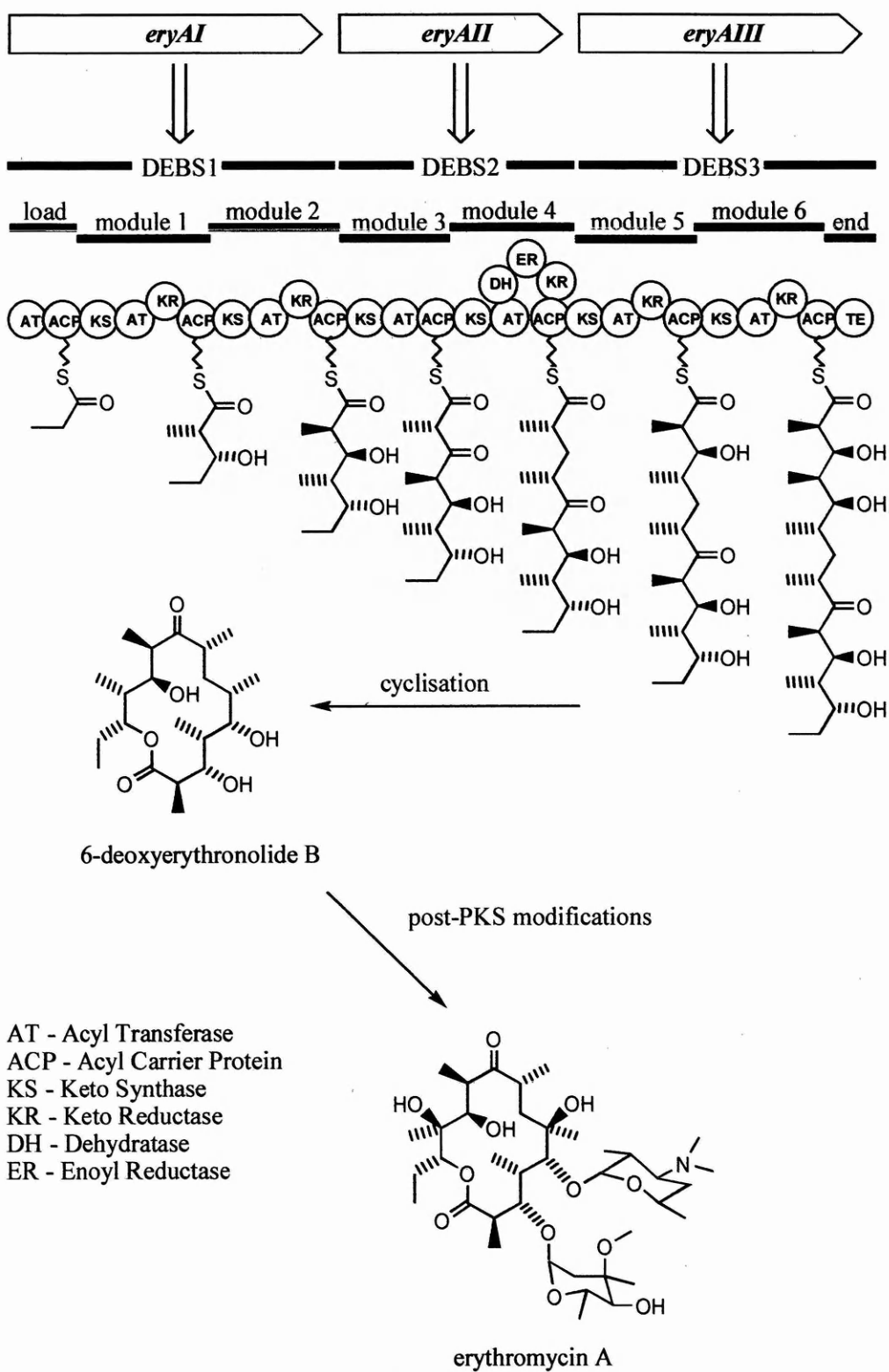
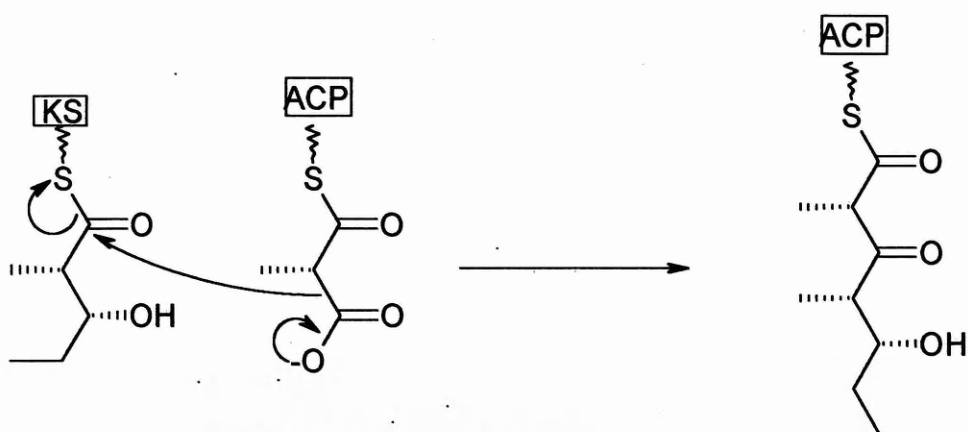


fig 1.1.3.Representation of erythromycin A biosynthesis



Beta keto is then reduced by KR domain

fig 1.1.4 Methyl-malonyl group loads onto ACP and is incorporated into chain

In order to investigate the underlying mechanisms that control stereochemistry and substrate specificity within these multifunctional enzymes, it is useful to have available a simplified form of a PKS. This can be achieved in the form of a triketide synthase, a truncated form of the erythromycin PKS consisting of one of the DEBS cassettes. The first triketide synthase to be genetically engineered was the so-called DEBS1-TE¹³ (fig 1.1.5).

DEBS 1-TE: The first modified PKS

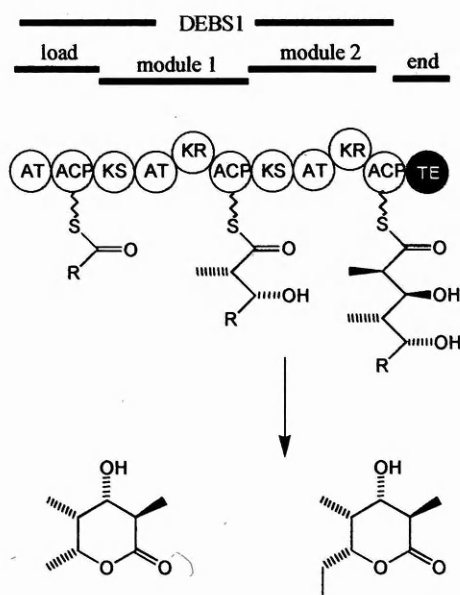


fig 1.1.5. The lactones produced from DEBS1-TE are known as the 'normal' lactones (normal stereochemistry)

This involves the DEBS 2 and 3 enzymes being removed and the thioesterase, the module thought to be responsible for the cyclisation of the lactone, being moved from the end of

the DEBS 3 enzyme and brought to the end of DEBS 1. This proved very successful and the resulting triketide was biosynthesised *in vivo* in quantities comparable to that of the original erythromycin. It was also shown that the stereochemistry of this resulting triketide lactone was also shown to have the expected stereochemistry corresponding to that area of the erythromycin PKS, as predicted by Celmer¹⁴ (see page 9). When a construct was made consisting of the DEBS 3 cassette with the loading domain from DEBS 1 and the ketosynthase from module 5 (KS₅) replaced by KS₁ (this construct forms an integral part of this thesis and will be discussed in more detail in the second section), it was found to produce the expected product in only very small amounts (<4mg/dm³). It was also seen to produce a range of other stereoisomers within the culture broth. Modules have also been successfully incorporated into DEBS derived systems.^{15,16} Modules or domains from the rapamycin and avermectin PKSs are sometimes used as 'spare parts' to modify DEBS derived PKSs. For example, when the domain AT from module 1 in DEBS1-TE is replaced by the AT from rapamycin module 2, a triketide lactone is observed where the methyl group at the 4-position is missing.¹⁷ This is because the AT₂ from the rapamycin PKS specifies a malonate extender whereas the DEBS AT₁ specifies a methylmalonate. Manipulation can also be carried out at the genetic level with regards to the functionality of the various domains. For example, the second ketoreductase module KR₂ in the aforementioned DEBS1-TE was deactivated. This had the effect of eliminating reduction at the 3-position of the resultant triketide lactone. Therefore, where a hydroxyl group would normally be seen, a keto group is now observed. Production levels of this triketide lactone were quite high but still less than expected.

The construction of these hybrid polyketide synthases opens many new doors in the field of genetic engineering of new type I PKS systems. The possibilities for new hybrid and truncated systems are almost limitless, leading to new antibiotics, immunosuppressants and other important compounds.

Since the triketide PKS DEBS-1TE was first genetically engineered, many other truncated PKSs have been engineered in order to produce a variety of different lactones, each of which requires both molecular characterisation and quantification. Quantification of these lactones is important since the efficiency of enzyme production is of fundamental interest.

When the macrolide antibiotics are displayed alongside each other, there can be seen to be a marked similarity between the stereochemistries of the alkyl and oxygen functionalities at any point along the chain (fig 1.1.6). This similarity, first noticed by Celmer, would indicate that each chain extension module extends the chain in an absolute stereochemical way. The stereochemical similarity between these sometimes quite complex macrolides seems to suggest that the polyketide synthases have similar properties and may have common evolutionary origins.¹⁴

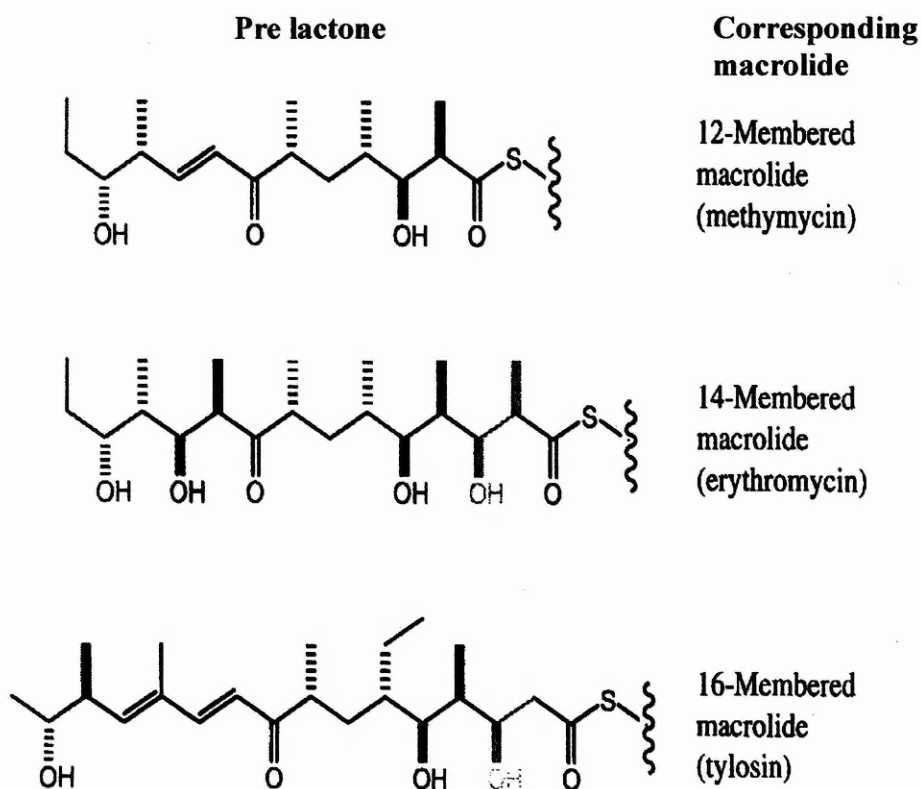


fig 1.1.6. Celmer first noticed stereochemical similarities along the chain

1.2 Mass spectrometry

In mass spectrometry there are three main stages that a sample must undergo before it can teach us anything useful. The first is the ionisation step. For this to occur, in most cases the molecules need to be in the gas phase (except where the volatilization process produces ions directly). Secondly, the resultant ions must be separated according to their mass. In practice however it is only possible to separate ions according to their mass to charge ratio (m/z) but we may assume in most cases that z , the integer charge, is one since multiply charged ions are rare compared to singly charged ones in the mass spectra of smaller molecules. We may therefore assume that the value of m/z is the mass of the ion.¹⁸

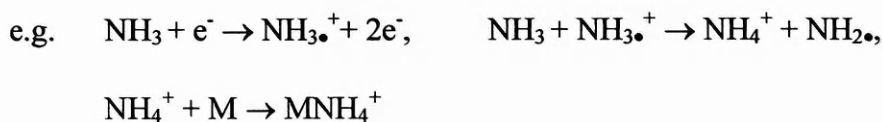
Ionisation techniques

Ionisation may be described in terms of its hardness. This refers to the extent to which fragmentation of the target molecule occurs during the ionisation process.

Electron impact (EI) consists of bombarding the sample with electrons, and is a so-called 'hard' ionisation method. This means that the electrons delivered to the sample are of such high energy (usually 70 eV) that the molecule spontaneously fragments and sometimes little or no molecular ion is formed. The decomposition of the molecular ion in this case can happen in a number of ways, from the low energy rearrangement of bonds, to the relatively high energy homolytic breaking of single bonds. EI is particularly useful for the analysis of smaller molecules which are relatively volatile since the sample must be in the gas phase. The EI source is easily interfaced with gas chromatography because the eluting compounds entering the source are already in the gas phase.

Chemical ionisation (CI) is by definition a softer ionisation technique in which the source region is flooded with a reagent gas. The reagent gas is by far in excess compared to the

sample. When the ionisation chamber is bombarded with electrons, the reagent gas molecules (being more numerous) are ionised first. The primary radical ions generated undergo ion-molecule interactions to generate even-electron species which in turn cause secondary ionisation of the sample molecules.



Using this method, there is a greater chance of observing the molecular ion.¹⁹ CI is often used as a complementary method to EI. Since these methods are limited to compounds in the gas phase, a heated inlet probe may be used to somewhat increase the range of the technique.

Electrospray ionisation (fig 1.2.1.) has, in recent years, opened new doors to the analytical chemist in terms of the analysis of high molecular weight compounds such as proteins and polymers with masses of over 100,000 Da.²⁰ This ionisation method allows good mass spectra to be gathered from very small molar concentrations. The main reason that the electrospray technique is so suited to large molecules is that it has the almost unique ability to produce multiply charged ions.²⁰ This series of very large, highly charged species may then be analysed by a large variety of mass spectrometers. The spectrum may be deconvoluted to give a molecular mass which is more accurate because it is an average value taken over a series of peaks. Samples ionised by electrospray must be in solution. This feature means that an electrospray source may easily be coupled to various types of liquid chromatography, the most common today being high performance liquid chromatography. Electrospray is a very 'soft' ionisation technique.²¹ This means there is little fragmentation and we may easily see the peak which corresponds to the molecular ion.

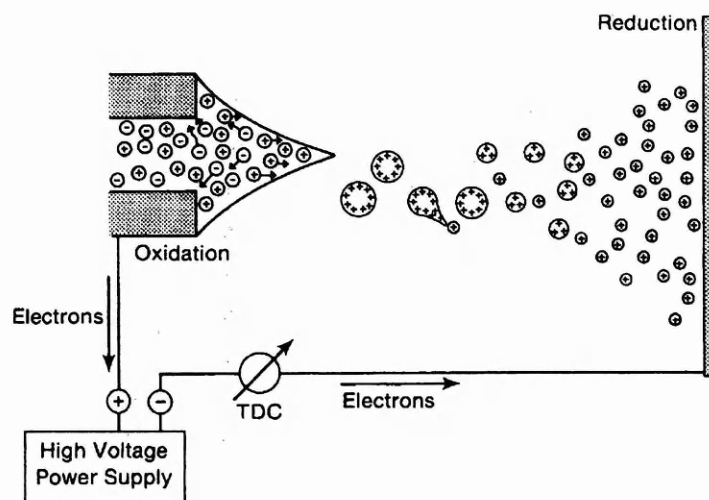


fig 1.2.1. Schematic of electrospray ionisation²⁰

A very high potential is applied to the tip of a needle, through which is 'sprayed' the sample solution. This high potential induces the same charge at the surface of the droplets. These droplets are then repelled from the needle tip towards an oppositely charged plate. As this happens, the solvent evaporates making the droplets smaller but with the same surface charge. This increased surface charge density causes repulsion at the surface. Eventually when the droplets reach their Rayleigh limit (the point at which Coulombic repulsion exceeds surface tension) the droplet will explode. This cycle is repeated until all the solvent is evaporated and we are left with single, solvent free, ions.²⁰

Mass analysis

Once the sample has been ionised, it must undergo mass separation or mass analysis. This may be achieved by a number of methods outlined below.

The basic theory behind **time of flight** mass spectrometry is probably the simplest to understand. All ions are extracted from the ionisation source (ionisation may take place in a number of ways) and accelerated by a pre-set potential difference. Because all the ions are subjected to the same potential difference, the kinetic energy to which they are then accelerated is proportional to the charge on the ion.^{19,18} The amount of time taken to travel

the length of the chamber, or field-free drift zone, is therefore proportional to the mass to charge ratio of the ion. One problem facing this method of mass analysis is that the ion should ideally have no kinetic energy to start with so that all the ions will be accelerated to the same kinetic energy. In practice however the nature of most ionisation methods means that the resultant ions are moving in a random manner with a range of kinetic energies. One way in which this problem may be overcome is by accelerating the ions in a certain direction along a tube so that only the ions of little or no kinetic energy in the direction perpendicular to the direction of motion reach the end of the tube. The ions may then be accelerated in the direction perpendicular to the motion along the field-free drift zone all with the same kinetic energy. Reflectron instruments are often used to effectively increase the path length of the field free drift zone and so increase resolution and sensitivity.

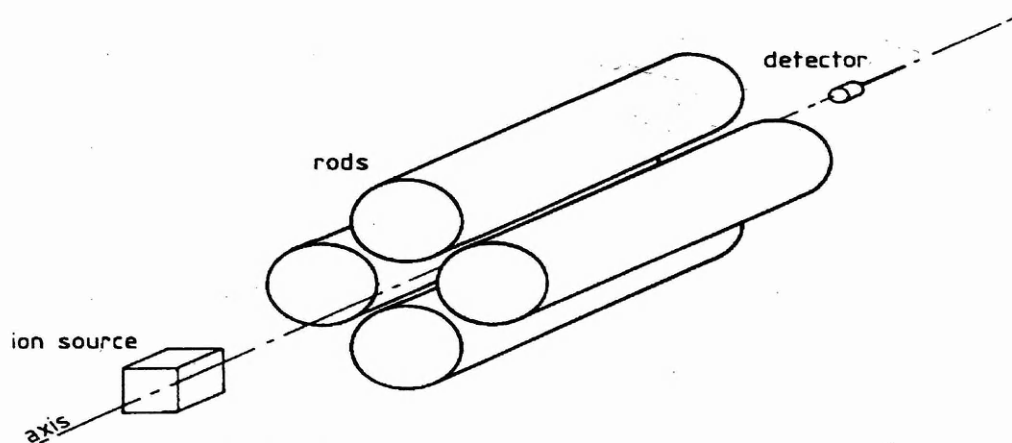


fig 1.2.2. Quadrupole mass analyser (opposite rods have similar charge)¹⁸

The **quadrupole** (fig 1.2.2) mass spectrometer works in a very different way to the conventional magnetic sector instrument (see later). Firstly, it does not require the ions to be accelerated to high energies in order to separate them according to their mass. Only a few volts are used to accelerate the ions before they enter the quadrupole. This consists of four parallel, cylindrical rods. Rods opposite each other are connected and a voltage applied between the two pairs of rods. An ion travelling down the centre of the rods will be repelled equally by the oppositely charged rods and so held in an energy minimum or

valley along that plane. However it will be attracted equally by the similarly charged poles in the other plane and so is at an energy maximum or hill along this plane. A combination of RF and DC voltages is applied to the poles. The amplitude of the motion away from the central path depends on the magnitudes of the RF and DC voltages and the mass of the ion. It is possible to tune the voltages so that ions of a desired mass reach the detector at the end of the quadrupole and are not lost to the sides.¹⁸ The quadrupole is not as accurate as larger sector instruments but has the advantage of being relatively cheap, small and ideal for routine bench-top analysis.

The **ion trap** analyser works by creating a field inside the trap similar to that of a quadrupole instrument. There are two end cap electrodes and one doughnut shaped ring electrode. However unlike the conventional quadrupole instrument, where the ions travel along the analyser to the detector at the end, the ions are trapped in a stable oscillating trajectory within the cell²². Then the various voltages across the electrodes are altered, and this has the effect of destabilising the trajectory of certain ions according to their mass. These destabilised ions are then ejected from the trap and detected²³.

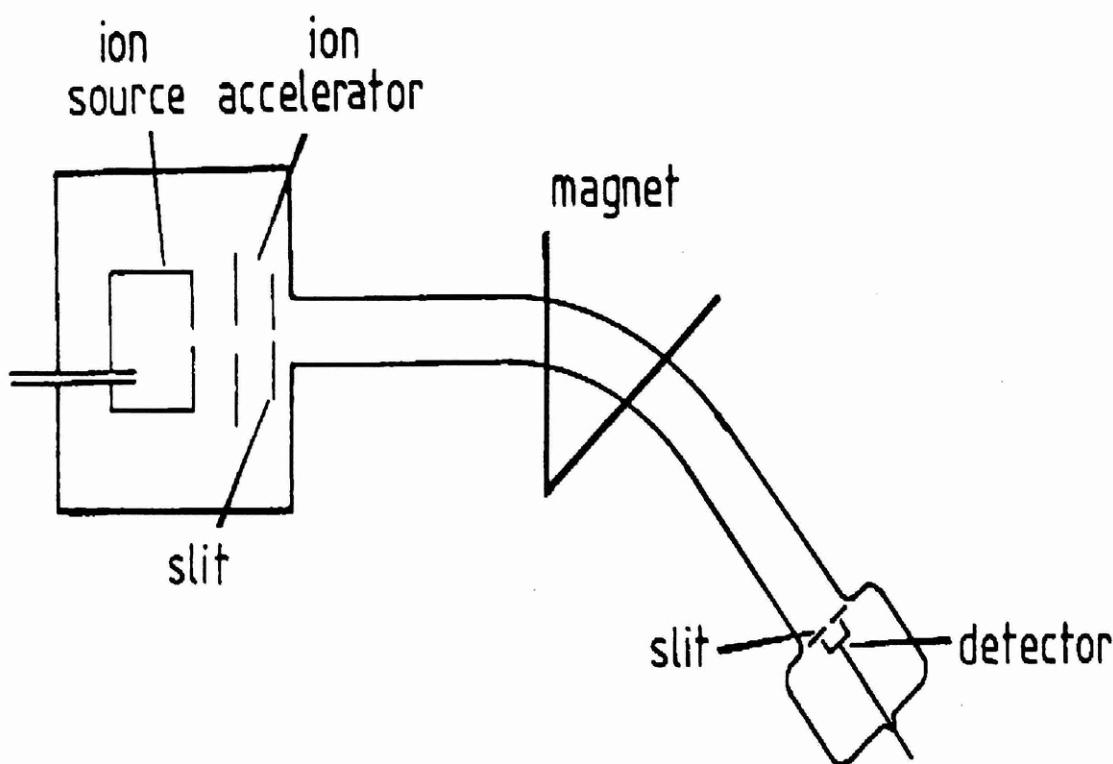


fig 1.2.3. Schematic of a single focusing magnetic sector mass analyser¹⁸

In the **magnetic sector** instrument (fig 1.2.3) ions are accelerated by a potential difference which may be as high as 10 kV. The ions are made to converge on the entrance slit of the analyser by a pair of focussing plates. The kinetic energy eV given to an ion is given by $eV = \frac{1}{2}mv^2$. So singly charged ions entering the analyser all have the same kinetic energy. However ions of different masses must be separated. This is achieved by applying a magnetic field perpendicular to the direction of motion. This magnetic field will exert a force on the ion perpendicular to both the original direction of motion and the applied magnetic field. The deflected ions will then travel in a circular motion of radius r . The value of r is proportional to the mass of the ion and also to the magnitude of the magnetic field B . So by varying B it is possible to separate out the ions according to their mass.²³

1.3 Gas Chromatography (fig 1.3.1)

Chromatography is the separation of mixtures of compounds by the partitioning or distribution of those compounds between two different phases²⁴.

The various compounds in a mixture are carried along by a mobile phase across a fixed stationary phase. The components partition to different extents and so are retarded by different amounts depending on their physical and chemical characteristics. The compounds with greater solubility in the stationary phase will be retarded to a greater extent and so will take longer to reach the detector. In capillary gas chromatography, the mobile phase is an inert gas and the stationary phase is usually a high molecular weight liquid, which is deposited onto the inner surface of a length of long fused silica capillary tubing

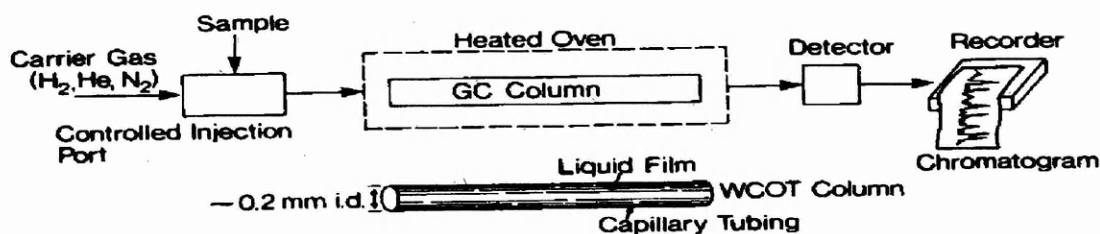


fig 1.3.1 A basic GC setup²⁴

Sample introduction

The first rule for introduction of the sample onto the column is that it must be in the vapour state. The most common method of achieving this is by the use of a self sealing septum, through which is injected the sample into a heated injection port. The sample, usually in solution is collected onto a sorbent material within the injection port (fig 1.3.2), is thermally desorbed, and introduced onto the GC column under the influence of the carrier gas. The step of thermal desorption is a very important one and must take place quickly and evenly²⁴. The sample needs to be introduced onto the head of the column as a thin

band and the sample injected must be a true representation of the original mixture, in terms of relative amounts of individual compounds. To allow the sample to be desorbed quickly the injection port will usually contain a small plug of silanised glass wool. This serves to increase the effective surface area of the injection port and so make sample desorption much quicker and more even. It is important that the injection port liner and glass wool are silanised and that the temperature is carefully monitored as active sites coupled with too high a temperature may facilitate sample degradation. The proper injection technique will serve to improve the quality of peak shape and so make quantification more accurate. It will also make the measurement of relative retention times of the compounds in the mixture more accurate.

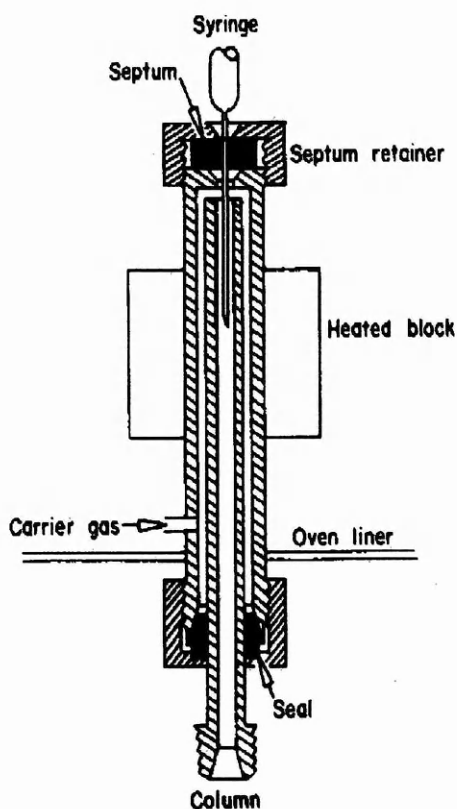


fig 1.3.2. Typical flash vaporisation sample injector²⁴

Sample criteria

The concentration and amount of sample which is loaded onto the head of the column is also very important. If the concentration of sample is too high then the column will become overloaded. This will cause 'fronting' where the peak tails towards the front. The sample amount for fused silica capillary columns needs to be no greater than about 100ng per component and preferably about 50ng per component to avoid column overloading. If, however, there is too much sample solution introduced, then the sample will not be vaporised homogeneously and there will be peak 'tailing' where the peak tails towards the rear. The usual volume for a normal splitless injection is about 1 μL . It is possible to inject up to 100 μL at a time using a large volume injector apparatus

Sample separation

Once the sample is vaporised and introduced onto the head of the GC column, it is carried along by the pressure of the carrier gas. This must be a relatively inert gas such as helium or nitrogen. The gas flow rate and pressure are carefully regulated by a series of valves and regulators. When the separated compounds reach the end of the column, they are detected by some means. The response of the detector plotted against the time taken to reach the detector constitutes the gas chromatogram. This chromatogram can give us information about the eluting compound. The peak retention time gives qualitative information and the peak itself can give quantitative information from its area²⁵. The flame ionisation detector is probably the most universally used, however the mass spectrometer, although more expensive, gives more information about the eluting compound. Whichever detection method is chosen for a particular piece of analysis, the detector must be insensitive to the carrier gas.

The split injector is a useful device as it allows the use of samples more concentrated than can normally be analysed effectively. The sample is vaporised in the injection port in the

normal way but is split unequally (normally about 100:1). The larger fraction is vented to waste while the smaller one is carried onto the column. However, if quantitative information is required then the exact split ratio must be known. This can be measured either electronically or by the more usual bubble-in-a-tube method.²⁴

The gas chromatogram is also an important indication of how efficiently the GC instrument is performing. Basic physical phenomena may be described by theoretical parameters that, when altered, can greatly improve separation and peak width.²⁴

There are three main parameters which go to make up equations that give information about the efficiency of the column. The first of these parameters to be measured is the elution time of an unretained peak t_m . This may be the solvent front or may be measured using a gas such as ammonia and a stopwatch. The gas is injected onto the column and the time taken to show on the mass spectrometer is measured. The second parameter is the retention time of the peak of interest t_i . This is simply read off the chromatogram. The third is the peak full width at half maximum FWHM w_i . This is a measurement, as the name suggests, of the width of the peak in seconds, at half the height. Column temperature, carrier gas flow rate and column can greatly effect the quality of the resultant chromatogram. By changing these in varying amounts it is possible to achieve the best results. But how is it possible to measure how well a chromatographic set up is performing? Using the simple measurements described it is possible to work out the number of theoretical plates N by the relationship:

$$N=5.54(t_i/w_i)^2$$

This value shows the column efficiency in producing narrow peaks. The smaller the peak spread, the larger the value of N and the more efficient the column.

The resolution R_{ij} between two peaks (how easily the column separates them) may be calculated from the equation:

$$R_{ij}=2[(t_j-t_i)/(w_j-w_i)]$$

Where t_j and w_j are the retention time and FWHM respectively of a second peak.

A useful equation to describe the efficiency of a column in terms of various physical properties was proposed by Van Deemter:²⁴

$$h = A + B/u + Cu$$

h is the column efficiency (lower number indicates higher column efficiency).

u is the carrier gas flow rate

A , B and C are various coefficients which make adjustment for molecular diffusion through the column, the effect of different path lengths along the column and mass transfer between the mobile and stationary phases.

If for example a plot is made of u against h , it may be seen that there is a certain value of u where h is at a minimum. This is the optimum flow rate for this column. It also shows how increasing the gas flow rate and so speeding up the experiment will affect the quality of the results (quality may sometimes need to be sacrificed in favour of time).

The Van Deemter equation as it is shown above was first derived when packed columns were in common use. An ideal case would be a column with no packing but with the stationary phase merely coated thinly onto the inner wall.

Later generations of the Van Deemter equation have been adapted for capillary GC columns and it can be seen from these that $h \propto r^2$ where r is the internal radius of the column. Therefore, the thinner the column, the more efficient the chromatography. Today, columns have internal diameters sometimes as small as 0.2mm. The limiting factor here is physically making them thinner and still having them work effectively.

Today, GC instruments can be bought relatively cheaply and can easily be coupled to most mass spectrometers. They are small and can easily fit onto a bench top, the columns are easy to use and change and are flexible enough to be coiled round and made to fit in the most awkward of spaces. Because the column is a capillary it may be connected to the mass spectrometer simply by inserting it directly into the source. With the advance of computers, data processing has also become a more simplified affair. Gas chromatograms can be zoomed in, mass spectral data can be accessed simply by clicking on the required peak and calibration plots can also be made automatically. Peaks can also be integrated electronically instead of the traditional method of cutting them out from the paper and weighing them. The GC instrument itself can now be fully controlled from the computer workstation. Individual methods containing mass spectrometer tune files, GC temperature programs, gas flow rate and many other parameters can be stored as a single program and can be recalled instantly. GCs have become very important analytical tools to everyone from the biologist to the synthetic chemist and almost all laboratories have access to one.

1.4 High Performance Liquid Chromatography

The basic principles of separation which lie behind high performance liquid chromatography (HPLC) are very similar to those of GC and, indeed, many other types of chromatographic separation techniques such as tlc. Many of the equations for HPLC, such as resolution and column efficiency, have much the same form as GC, as might be expected. However in this discussion, it is mainly the differences that will be dealt with, as these are what allow HPLC to be used beyond the usual limits of GC.

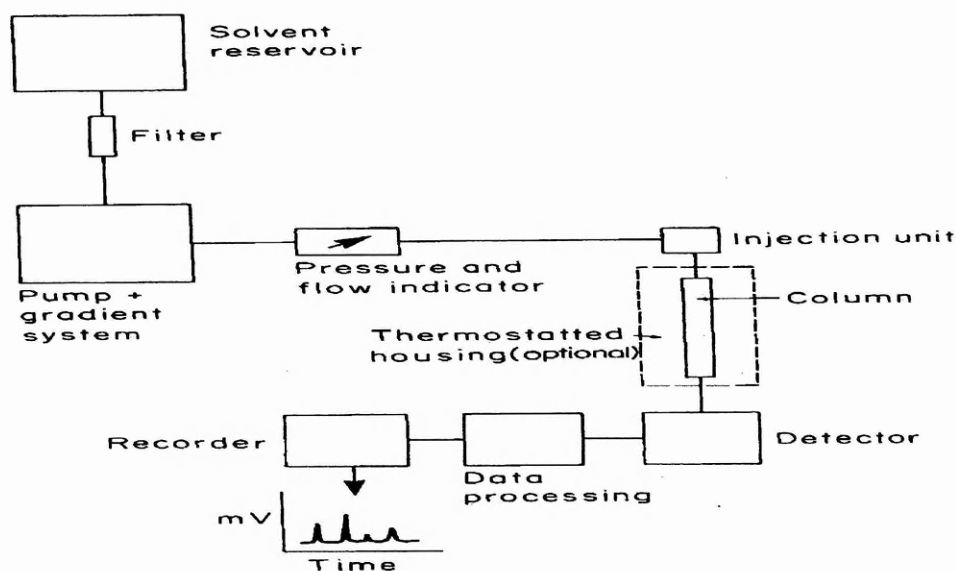


fig 1.4.1. A block diagram of the workings of an HPLC system²⁶

In HPLC, the mobile phase becomes more important as interaction between it and the solute is more selective. In GC, however, all the sample vapours are soluble in all proportions because the mobile phase is a gas. This makes HPLC more selective and more able to perform more difficult separations.²⁷

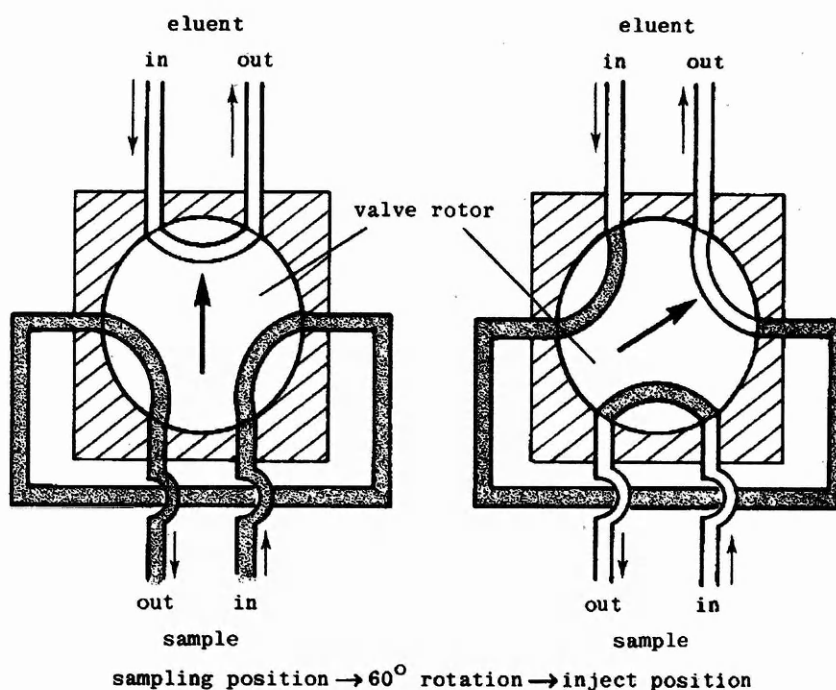


fig 1.4.2. An HPLC sample loop valve. The sample solution is placed directly into the solvent flow²⁶.

Sample introduction

The sample in liquid solution is introduced onto the head of the column. Because of this, the type of sample which may be loaded is only limited to how easily it can be dissolved. Most substances will dissolve in something so this is not usually a problem, however if the sample is to be a true representation of the original then a solvent must be chosen into which everything of interest will dissolve. Because there is no thermal desorption, there is no danger of decomposition so thermal stability and volatility are not factors. This means that unlike GC, HPLC may be used for a very wide range of compounds right up to proteins with molecular weights of many thousands. It can also be used for compounds that are thermally quite unstable²⁸.

The sample is usually applied to the column by means of an injection loop (fig 1.4.2). This consists a loop which may be filled with a set volume of analyte solution which is switched

by a valve. This places the loop directly into the flow of the solvent and the solution is washed straight onto the column.

The solvents are pumped from the solvent reservoir into a mixing chamber. When the solvents meet each other at high pressure (high pressure mixing), dissolved gases can come out of solution causing bubbles to form. These bubbles can have a disastrous effect on the separation and retention times of eluting compounds and can ruin expensive columns. This can usually be resolved by degassing solvents properly before use. Solvent sparging is the most common method. This involves bubbling a gas such as helium through the solvent for a time before use. Another way is by sonication, and a third is by filtering the solvent under a vacuum. When the solvent mixing chamber is set up before the pump (low pressure mixing) then this problem is greatly reduced although degassing is still advisable.

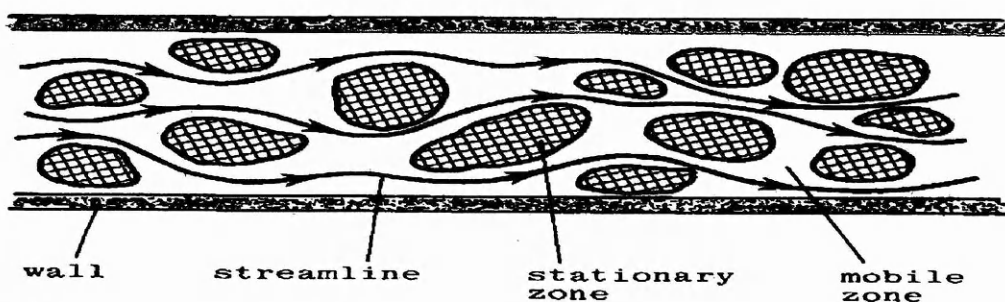


fig 1.4.3. A cross sectional view of an HPLC column²⁷

Column and packing

The HPLC column (fig 1.4.3.) is a precision piece of equipment. It is generally a piece of stainless steel tubing 10 to 25cm in length and about 0.5cm in diameter depending on the application. The packing is made of small supports with an average diameter of about 5 μ m, onto which the stationary phase is coated. The most common coating is probably octadecylsilane or ODS; this is also known as a C-18 column.²⁹ Silica is also used as a stationary phase. The fact that the particle size is so small and that the particles are packed in so tightly (pressures used to pack columns are sometimes in excess of 700bar) means that the solvent pressure through the column must be high (about 150bar).

Reverse and normal phase

The ODS stationary phase mentioned earlier is used in what is known as reverse phase HPLC. This means that the stationary phase is of low polarity and the mobile phase is of medium to high polarity. The solvent used here is usually water (or buffer) mixed with methanol or acetonitrile (either isocratically or as a gradient). This means that the compounds which are most polar will be eluted first and in order to increase the retention time of the solutes one must increase the mobile phase polarity. In normal phase HPLC, silica columns are often used, giving a highly polar stationary phase. The mobile phase polarity must therefore be low to medium. For this hexane or heptane are usually used in conjunction with chloroform or dichloromethane. The solutes elute in the opposite order to that of reverse phase HPLC and to increase retention time one must decrease the mobile phase polarity. Reverse phase utilising a C-18 ODS column is by far the most common HPLC set-up today. This is because reverse phase HPLC allows samples with a wide range of polarities to be analysed. It is also generally easier from an experimental point of view, being faster and more reproducible than normal phase. However reverse phase is not without limitations. Firstly, it is only suitable for pH conditions of between about 3 and 8. Outside these limits the stationary phase starts to degrade. Secondly, the peak shape and reproducibility are sometimes not good because of the 'bleed' of unreacted silanol groups from the column. Thirdly, the mechanism for reverse phase retention is not fully understood, and this can cause problems when attempting to optimise separation from a theoretical point of view.

One of the most useful applications of HPLC is the ability to carry out precise preparative separations.³⁰ Corresponding preparative columns are usually commercially available for most analytical columns. The ideal is that only the flow rate need be adjusted to obtain the same results as the corresponding analytical column but on a larger scale, but it is not

always as simple as this and a certain amount of adjusting of various parameters is usually appropriate. The fact that HPLC is so precise, and that it is very rare that two peaks cannot be resolved given a little investigation means that even small quantities (sometimes less than 1mg) of a mixture of compounds with very similar retention times may be separated and collected for further analysis such as NMR spectroscopy. An important consideration when performing preparative HPLC is that it should be looked on as a final, fine isolation method. That is, the compound or compounds of interest must be as pure as possible before the preparative HPLC step is carried out, especially if the impurities are in larger quantities than target compounds. This preliminary purification can usually be achieved by some other more crude means of LC, such as a flash silica column or size exclusion LC. In preparative HPLC, if a solvent is chosen where some of the impurities are insoluble then this can in itself act as a kind of cleanup step (as long as the sample is filtered before analysis).

Detectors

A good detector must have high sensitivity, because of the problem of column overloading, and it must be able to distinguish the solute from the noise at very low concentrations. It must also show a good linear response. That is, it must show a response that is directly proportional to the amount or concentration of solute eluting from the column.³¹

Probably the most commonly used detector is the UV/VIS absorbance detector. The eluant passes through the small flow cell and the solutes are detected according to their absorbance. This detector will however, only detect solutes that absorb in the UV/VIS region (i.e that have a chromophore such as an aromatic ring or conjugated multiple bonds) at a fixed wavelength. Compounds with no active chromophore will not be detected. The diode array detector is a more advanced descendant of the single wavelength uv detector. It allows a range of wavelengths to be scanned at any point in the experiment and so makes the detection more universal. As in GC, the mass spectrometer is a more useful detector. It gives mass spectral data on every detectable peak eluted rather than simply indicating that the peak is there, as most other detectors do. Unlike GC however, the types of mass spectrometer which may be used are limited. In most mass spectrometers, the sample must be in the gas phase before ionisation may take place. Because of the nature of HPLC and the fact that it is useful for larger, more involatile analytes, this is not always possible. The most attractive option for ionisation is therefore the electrospray ionisation source. This can give soft ionisation of large molecules directly from solution, perfect for coupling to HPLC.

1.5 Size exclusion chromatography

Size exclusion chromatography, or gel permeation chromatography (GPC) as it is sometimes known, is, in essence, the separation of molecules according to their size. It differs from almost all other forms of chromatography in that separation does not rely on any kinds of physicochemical properties of the molecule, the stationary phase or the mobile phase (other than that the samples must be soluble in it) but purely on the size of the solutes in relation to each other. Separation is achieved by means of small porous particles or beads (fig 1.5.1). When the sample mixture is introduced to the column, molecules which are too large to enter the pores are excluded and pass along the column unretained. Molecules which are small enough enter the pores and are retained for a time before being eluted along the column (fig 1.5.2). Thus, mixtures of molecules are separated in order of their size.

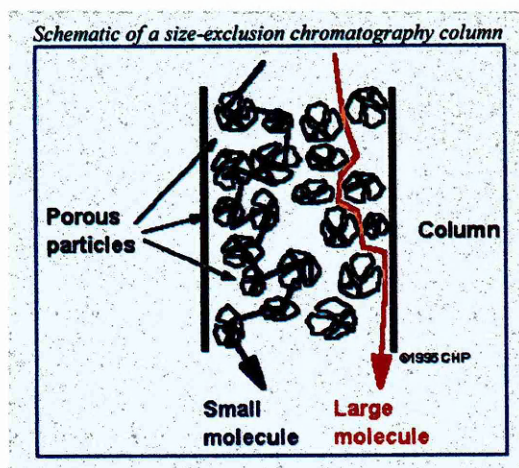
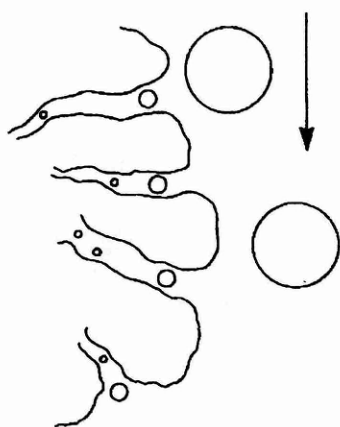


fig 1.5.1 Diagram of a size exclusion bead and fig 1.5.2 packed column

When a biological culture is extracted into a solvent, along with the compounds of interest (in this case triketide lactones) comes a large amount of other undesirable contaminants. This may be from the growth media or cell matter such as lipids or protein. Before the target compounds may be analysed effectively, this unwanted material must be removed. In employing size exclusion chromatography certain assumptions are made. Most

important is that most of the contaminants (for example starches and lipids) are much larger in size than the lactones of interest.

The column was packed with bio-beads obtained from Bio-Rad. These are porous styrene-divinylbenzene beads with an exclusion limit of 1000 Da. The lactones to be separated have a mass of 158-172 Da. The column was glass and was 1.5 m in length by 3 cm internal diameter. The beads were first swollen in dichloromethane, then poured into the column and eluted with dichloromethane. Once everything was set up, the crude extract was introduced to the column and pumped through with dichloromethane using a flow rate of 5 ml/min. The eluant was detected by a uv detector set to 280 nm and collected in 20 ml fractions. The fractions were analysed by GC/MS and the lactones were found to be exclusively within two fractions. When these fractions were concentrated and run on the HPLC they were seen to be noticeably cleaner than before the size exclusion chromatography. The semi-pure fractions obtained from the GPC were then combined, concentrated to dryness and dissolved in acetonitrile:water mix ready for preparative HPLC.

1.6 Objectives

The main aim of this study is to investigate the epimerase activity of the erythromycin KS1 when it is placed in an unnatural location at the beginning of the DEBS3 truncated PKS system. In order to accomplish this it will be necessary to identify, quantify, isolate and characterise the novel triketide lactones biosynthesised by the system.

The lactones will be identified by GC/MS techniques. Two methods of quantification will be evaluated. GC/MS and HPLC/MS methods will be compared with respect to limits of detection, linear dynamic range and reproducibility.

Methods of extraction and isolation of the lactones will be developed. A main focus of this work will be the development of preparatory scale reverse phase HPLC/MS using a FractionLynx mass spectrometer (Micromass, Manchester, UK.) with automated mass-dependent fraction collection.

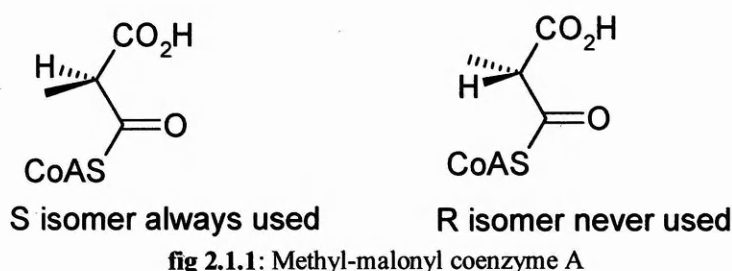
Initial characterisation of the lactones will be attempted by the use of spiking studies in which samples are analysed by GC/MS with and without the presence of known lactone standards. It may be necessary to synthesise a lactone standard if one is required and is not available. Then it will be necessary to use NMR spectroscopy, coupled with molecular modelling studies, in order to investigate stereochemistry of the biosynthetic products.

Chapter 2

Results and Discussion

2.1 Ery Load-KS₁-DEBS3, the reason for this particular construct.

In preliminary experiments on the mechanism of stereocontrol, it was proved that (S)-methylmalonyl CoA was used in both chain extension units of DEBS1. In contrast, (R)-methylmalonyl CoA was not utilised^{32,33} (fig 2.1.1). This showed conclusively that the control of stereochemistry at the methyl branching centres was not exercised by selection of the appropriate chiral form of the chain extension building block as had been proposed on the basis of *in vivo* experiments. Instead, the control must be exercised in the mechanisms of the chain extension processes themselves.



Subsequently, experiments were carried out with a labelled version of methylmalonyl CoA in which deuterium was located at the chiral centre³³. The hydrogen isotope label was retained at the methyl-branching chiral centre in the chain extension residue produced by module 2 but was completely lost in the chain extension process carried out by module 1.

These experiments also showed that the chain extension reaction was carried out with inversion of configuration in module 2. It was suggested, to explain the loss of the hydrogen from the chiral centre in module 1, that the equivalent condensation step might also take place in the same stereochemical sense with inversion of configuration to give the (2R)-isomer of the keto ester and that this isomer would then be epimerised to the (2S)-isomer by enolisation prior to reduction by the KR domain (fig 2.1.2).

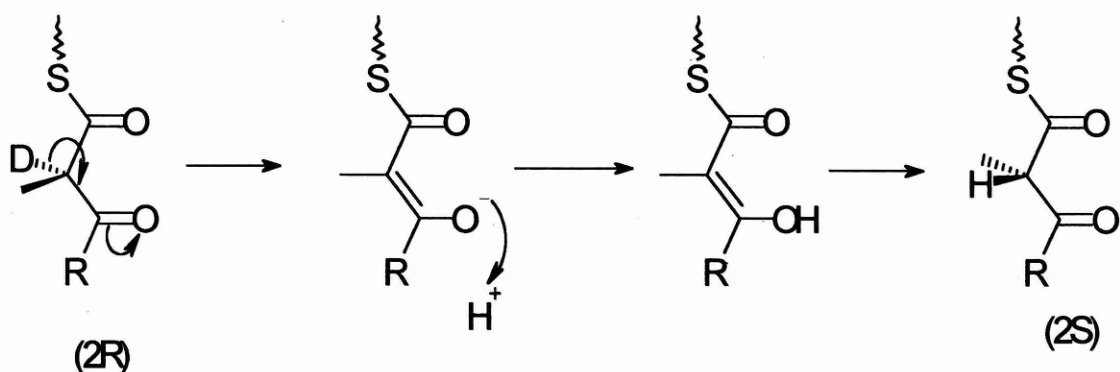


fig 2.1.2: Inversion of configuration and loss of deuterium proceeds by enolisation

These results demonstrate that module 1 holds the key as far as epimerisation is concerned.

What remained to be established after these experiments was the factor in module 1 which is responsible for the epimerisation process. We suspected that it might be the KS domain but, of course, it could have been any of the other domains or even a part of the protein in the reductive loop, which has not been assigned any specific function. In order to test the possibility that KS₁ might be an active participant in the epimerisation, a hybrid polyketide synthase was designed based on DEBS3 in which the first KS, KS₅, has been replaced by KS₁, and, to make the construct completely efficient, the loading domain was also incorporated at the front of the truncated PKS. This is shown in figure 2.1.3 and for comparison the reported⁴ pattern of behaviour of modules 5 and 6 in an unmodified version of DEBS3 is shown in figure 2.1.4.

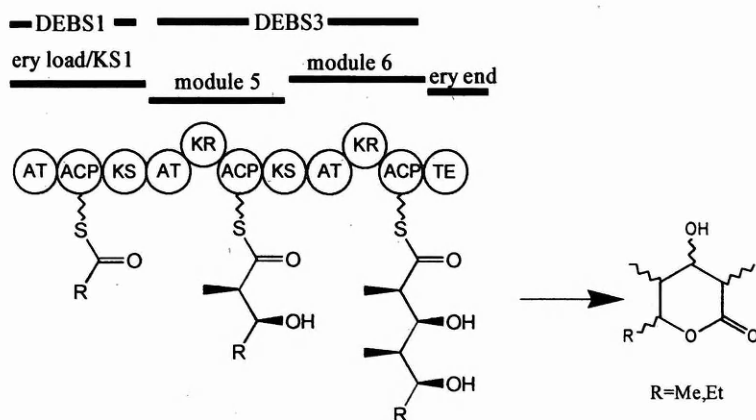


fig 2.1.3 DEB3 incorporating loading domain and KS1 from DEBS1

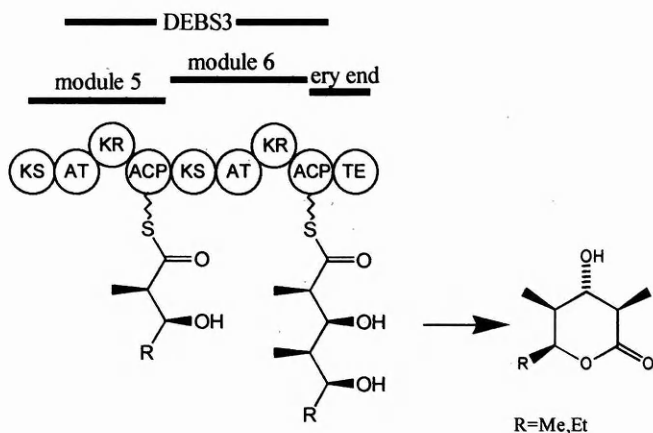


fig 2.1.4 Pattern of behaviour previously reported

Previous results suggested that one or both of the KR domains in DEBS3 show a low degree of stereospecificity in carrying out reduction of the keto group of a model keto ester. This observation raised the interesting possibility that one or both KR domains might accept both stereoisomers of the keto ester form of the diketide intermediate and so provide a very effective approach to probe the seat of the epimerisation activity. Note that only one stereoisomer (udu, see below) was reported by the Stanford group in *in vitro* studies with the unmodified version of DEBS3.⁴ This lacked a normally functioning starter domain and KS₅ in place of KS₁, so it does not provide a direct basis for comparison with any results from our own experiments.

Naming

Fig 2.1.5 below shows the simplified naming convention used in this report

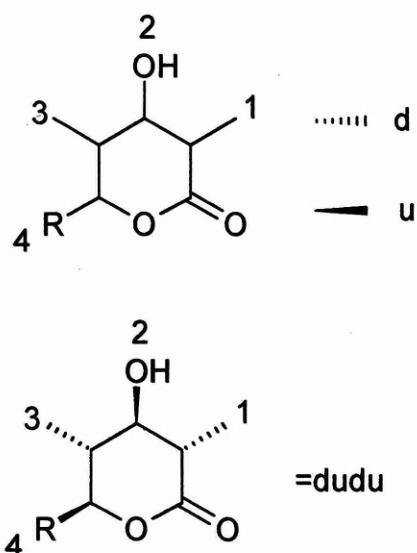


fig 2.1.5 shows how the stereochemistry of triketide lactones will be specified within this report. With the ring in the plane of the paper and the carbonyl group to the right, each substituent starting at 1 and going anti-clockwise will be either u or d.

2.2 Quantification study

When studying the nature of truncated or other genetically engineered polyketide synthases, a good indication of how well or efficiently the new synthase is performing is by accurate quantification of the metabolite product. If, as in the case of *S.erytharea* wild type, the product is produced in the order of grams, then it is probably enough to isolate the product on a silica column and simply weigh it. However, if, as is often the case with new constructs, the product or products are produced in the order of milligrams or sometimes even less, then accurate quantification by GC or HPLC is required. The question remains, which of the two methods is better for the analysis of triketide lactones, in terms of linearity across a good number of orders of magnitude, lower detection limits and reproducibility. The two instruments used within this study were the Finnigan-Matt GCQ, a GC coupled to a quadrupole ion trap with CI source, and a Hewlett Packard 1100 HPLC system coupled to the LCQ quadrupole ion trap with electrospray source.

A weighed synthetic triketide lactone standard of 2.2mg/ml was made up in methanol and serial dilutions were made from that stock using calibrated Gilson pipettes. The solution was diluted by half each time. These standards were then run on the LCQ and GCQ and the peak areas recorded (tables 2.2.1 and 2.2.3) and plotted against concentration of standard (figs 2.2.2 to 2.2.4).

table 2.2.1

Observed peak areas for varying concentrations of standard on LCQ

LCQ Calibration standards	
Conc(mg/ml)	Peak area
2.2	2.9563×10^7
1.1	1.4795×10^7
0.55	7.5852×10^6
0.28	3.8875×10^6
0.14	1.9548×10^6
6.9×10^{-2}	9.5483×10^5
3.4×10^{-2}	4.6209×10^5
1.7×10^{-2}	0
8.6×10^{-3}	0
4.297×10^{-3}	0
2.148×10^{-3}	0

fig 2.2.2

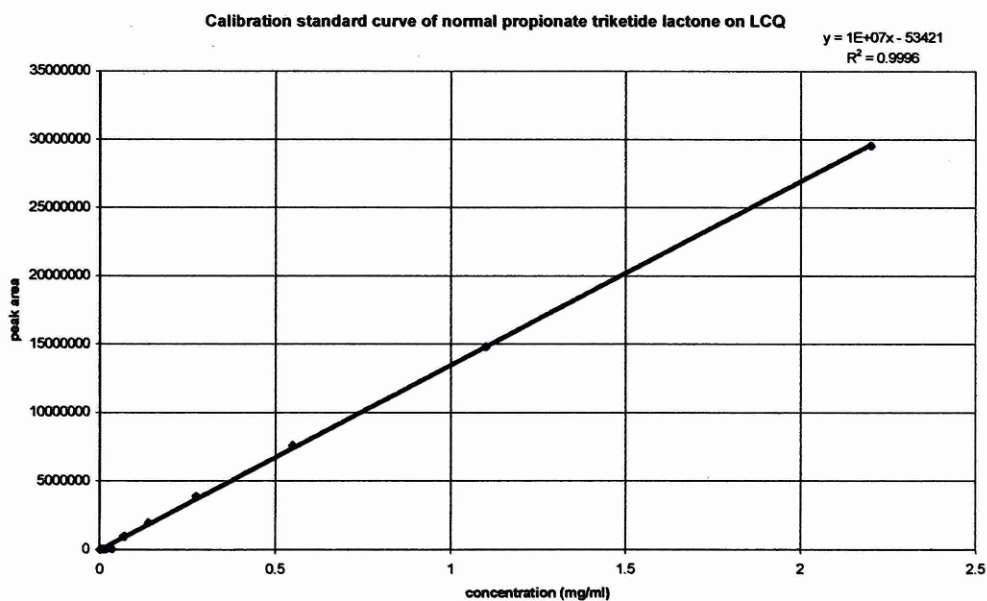
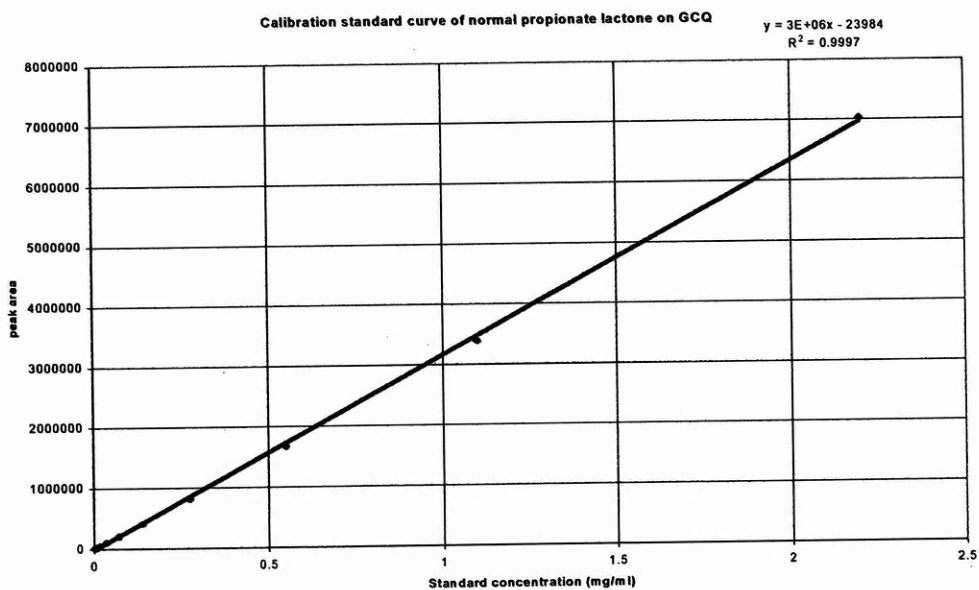


table 2.2.3

Observed peak areas for varying concentrations of standard on GCQ

GCQ Calibration standards	
Conc(mg/ml)	Peak area
2.2	7.0250×10^6
1.1	3.3922×10^6
0.55	1.6719×10^6
0.275	8.1644×10^5
0.1375	4.0922×10^5
6.875×10^{-2}	2.0022×10^5
3.4375×10^{-2}	1.0106×10^5
1.7188×10^{-2}	5.1028×10^4
8.597×10^{-3}	2.5614×10^4
4.297×10^{-3}	1.2698×10^4
2.148×10^{-3}	8.518×10^3

fig 2.2.4



The first significant thing to be noticed from the tables of results is the fact that the LCQ could not detect concentrations as low as could the GCQ. This is as expected because the lactone has the ideal structure to be detected by GC-MS (small and of ideal volatility) whereas the LCQ is more suited to larger molecules. It is clear that both instruments show

good linearity with R^2 values of 0.9996 in the case of the LCQ and 0.9997 in the case of the GCQ. The GCQ however, shows this excellent linearity down to lower limits than the LCQ and over several orders of magnitude.

From these results, it is clear that the GCQ is the better choice for both the quantitative and the qualitative analysis of triketide lactones. Another question however is that of reproducibility.

The same standard concentration of 4.297×10^{-3} mg/ml was injected ten times and the response factor was recorded (table 2.2.5):

table 2.2.5

Repeat injection of 4.297×10^{-3} mg/ml	
Injection N ^o	Peak area
1	12605
2	12337
3	12332
4	12132
5	12095
6	12719
7	12755
8	12382
9	11566
10	12452

The mean value of the peak area is $\bar{x}=12338$ where:

The sample standard deviation $s = 331.5$

i.e. a sample standard deviation of about 2.7% at a concentration slightly above the minimum detection limit.

From this it is clear that the GCQ is a perfectly accurate and reliable instrument on which to quantify triketide lactones.

The next step is to quantify the amount of lactone actually produced by a construct, in this case p015-5624 (Ery Load-KS₅-DEBS3). The plates were prepared as described earlier with a final extract volume of 30ml. A 1 μ l splitless injection was made onto the GC straight after the standards (in fact within the same run). The peak area of the main propionate peak taken from the mass 190 single ion chromatogram was measured and found to be 228601. If we use the equation given for the calibration curve on fig 2.2.4:

$$Y=3\times10^6\chi-23984$$

Where χ =concentration (mg/ml)
Y=peak area

then the concentration χ is calculated to be 8.42×10^{-2} mg/ml. There are 30ml of extract so the total amount produced is $8.42\times10^{-2} \times 30 = 2.53$ mg. The total volume of the plates was 1.6l (8 \times 200ml) so this particular construct produces the major triketide lactone on plates at 1.58mg/l. This is a significantly small amount making isolation and characterisation an extremely difficult (but not impossible) task.

The value for the Y intercept (-23984) gives an indication of the error associated with these experiments.

2.3 A "Spiking" study

Plates of the construct p015 5624 (ery load-KS₁-DEBS3) were extracted as described in section 3.2. This crude extract was analysed by GC-MS (temperature gradient 40°C-145°C in 23 mins) on a 30m, 0.25mm ID Rtx-5MS capillary column (Thames Restek UK LTD, Windsor, UK) for the presence of triketide lactones. From what is known about the polyketide synthase and how it builds up these metabolites, and also from previous studies⁴, we can predict that the expected product from this construct should be uduu. (fig 2.3.2) shows the gas chromatogram total ion current in the top line, the single ion chromatogram of mass 190, which corresponds to the propionate triketide lactone plus ammonia, (these lactones are detected as ammonium adducts as ammonia is the chemical ionisation gas used) is shown in the middle trace and the 176 single ion chromatogram at the bottom which corresponds to the acetate lactone plus ammonia. In the centre chromatogram corresponding to the 190 single ion chromatogram, there can be seen three peaks, one large one at 14:19 mins, and two smaller ones at 14:44 mins and at 15:04 mins, all of which have a mass spectrum typical of a propionate triketide lactone. These three peaks must therefore be stereoisomers of the propionate triketide lactone. This tells us straight away that the triketide synthase is behaving in a way that was unexpected.

It was important to try to determine the structure of these lactones in terms of stereochemistry. The first step was to try to match them with synthetic standards. This was done by spiking the chromatogram with a quantity of a synthetic triketide lactone standard (prepared by other members of the research group) of known stereochemistry. The second chromatogram (fig 2.3.3) shows the same crude extract but with 10µl of a 2.2mg/ml solution of the propionate lactone uddd added. This clearly shows an enhancement of the peak (2), which can be clearly seen in the 190 chromatogram (middle trace) for that

experiment at 14:46. This leads us to believe that the second peak is uddd or its enantiomer.

Further spiking studies of this nature using other synthetic standards (previously synthesised by other members of the research group) has allowed the elimination of other possible stereoisomers.

A second culture was extracted in the usual way (as described in section 3.2) but this time the mass 190 single ion chromatogram showed that there were two major propionate lactone peaks corresponding to peaks (1) and (3) of the first culture with regard to the retention times. Five synthetic standards were available (synthesised previously by other members of the research group except for duuu, the synthesis of which is described in sections 2.7 and 3.4) and these were spiked one at a time. The first chromatogram of the crude material was spiked with uddd. This showed an enhancement of peak (2). The second spike of udud showed an extra peak allowing it to be eliminated. The third spike of dudd showed an enhancement of peak (1). This tells us that peak (1) is the dudd isomer or its enantiomer. The fourth spike of stereochemistry duud showed the appearance of an extra peak allowing this one to be eliminated. The final spike of duuu showed an enhancement of peak (2). An attempt was not made to isolate peak (2) as it was thought that the amounts produced were too low. Peak (1) and the synthetic standard dudd were successfully separated using a chiral column indicating that the first peak is the expected DEBS 3 triketide lactone uduu, the enantiomer of the standard dudd. To summarise, the first peak is uduu and the second peak is duuu or uddd and the third peak is not udud, dudd, duud or duuu or their enantiomers (Table 2.3.1). So the question remains, what is the peak (3)? The first peak is clearly the expected DEBS 3 stereochemistry but the synthase is obviously producing something else. The next step was to attempt to isolate peaks (1) and (3) to verify their structure and stereochemistry.

Table 2.3.1

Lactone Standard	Observation
uddd	Enhancement of peak (2)
udud	Appearance of extra peak
dudd	Enhancement of peak (1)
duud	Appearance of extra peak
duuu	Enhancement of peak(2)

fig 2.3.2 Unspiked crude.

Chromatogram Plot D:\DOMINIC\DK226_1 Date: 06/22/98 11:55:47
 Comment: p015 5624 debs 3 crude extract at approx.25 ml
 Scan No: 802 Retention Time: 18:25 RIC: 459 Mass Range: 80 - 434
 Plotted: 297 to 901 Range: 1 to 1208 100% = 52717
 9.23%

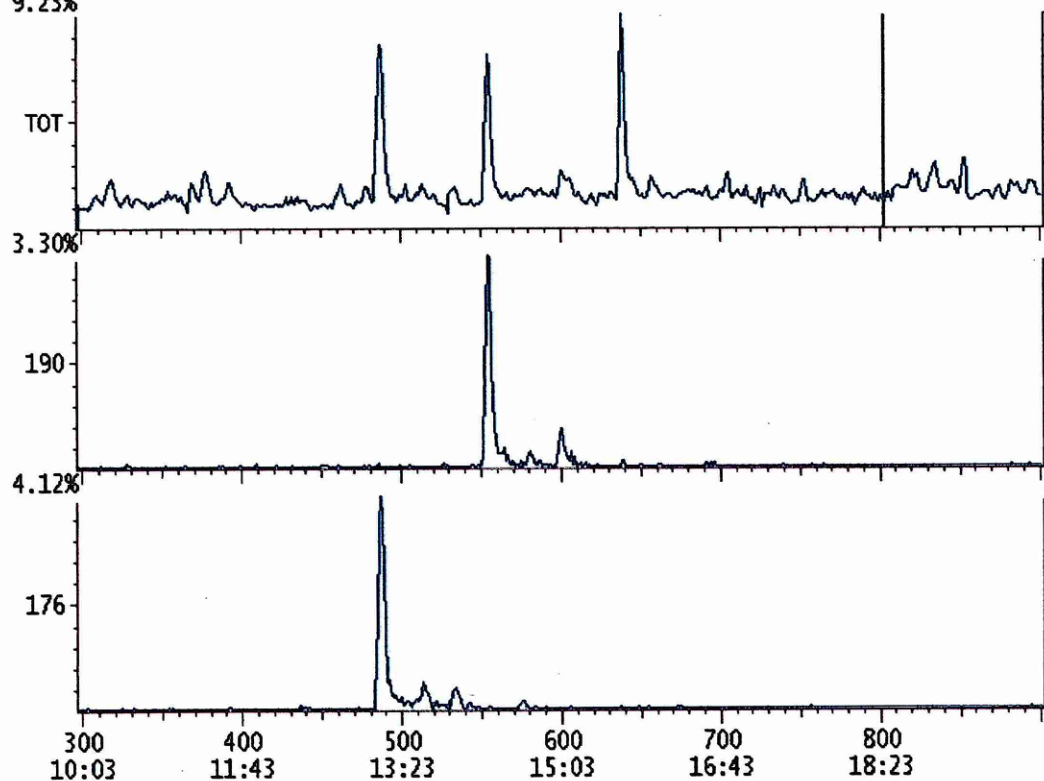
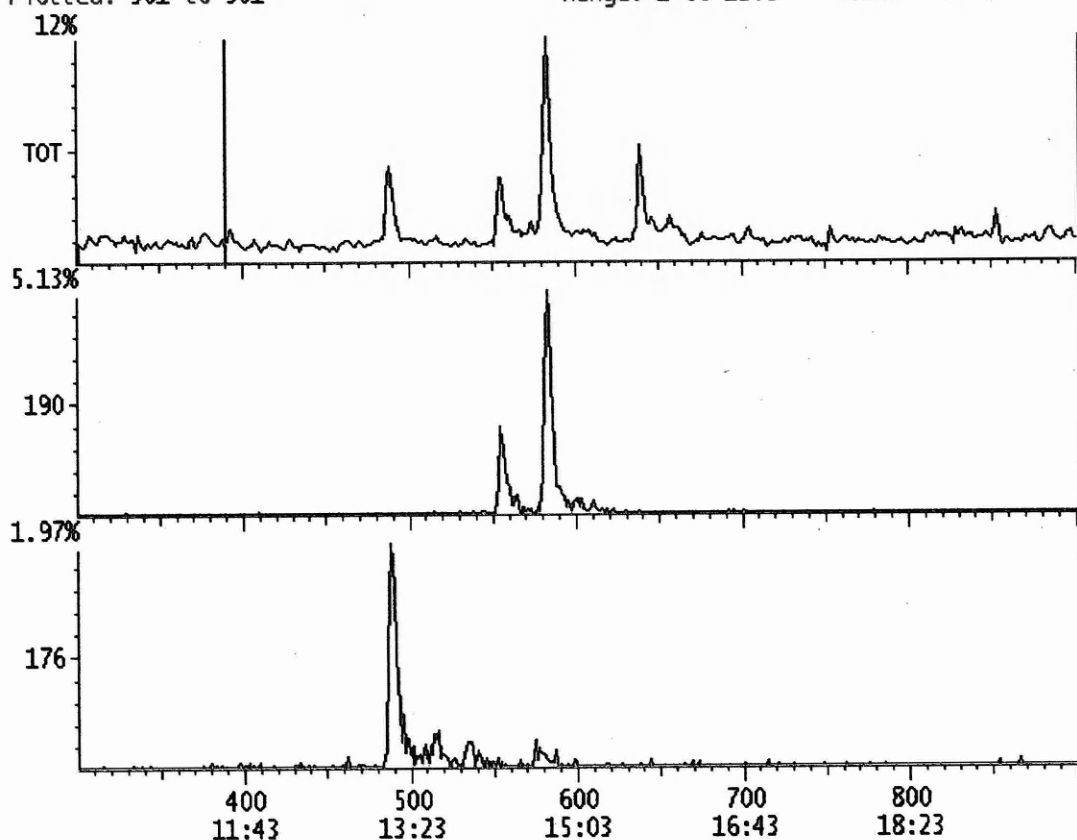


fig 2.3.3 Spiked with normal propionate lactone (uddd)

Chromatogram Plot D:\DOMINIC\DK226_2 Date: 06/22/98 14:03:23
Comment: p015 5624 debs 3 crude 25 ml with 10ul (10ul 2.2ug/ml norm lac)
Scan No: 390 Retention Time: 11:33 RIC: 327 Mass Range: 54 - 438
Plotted: 301 to 901 Range: 1 to 1208 100% = 46409



2.4 Preparative HPLC

Initially, all preparative work was carried out on the HP 1100/LCQ. There were however, many problems associated with this. The HP 1100 is primarily an analytical instrument, mainly because it is only capable of delivering a maximum flow rate of 5 ml/min. When the preparative column was fitted, which should be used at 20 ml/min, peak shape and resolution were severely compromised. The length of the run was doubled to try to improve separation but this was still unsatisfactory. It should be noted that the ions detected fly by electrospray as MH^+ .

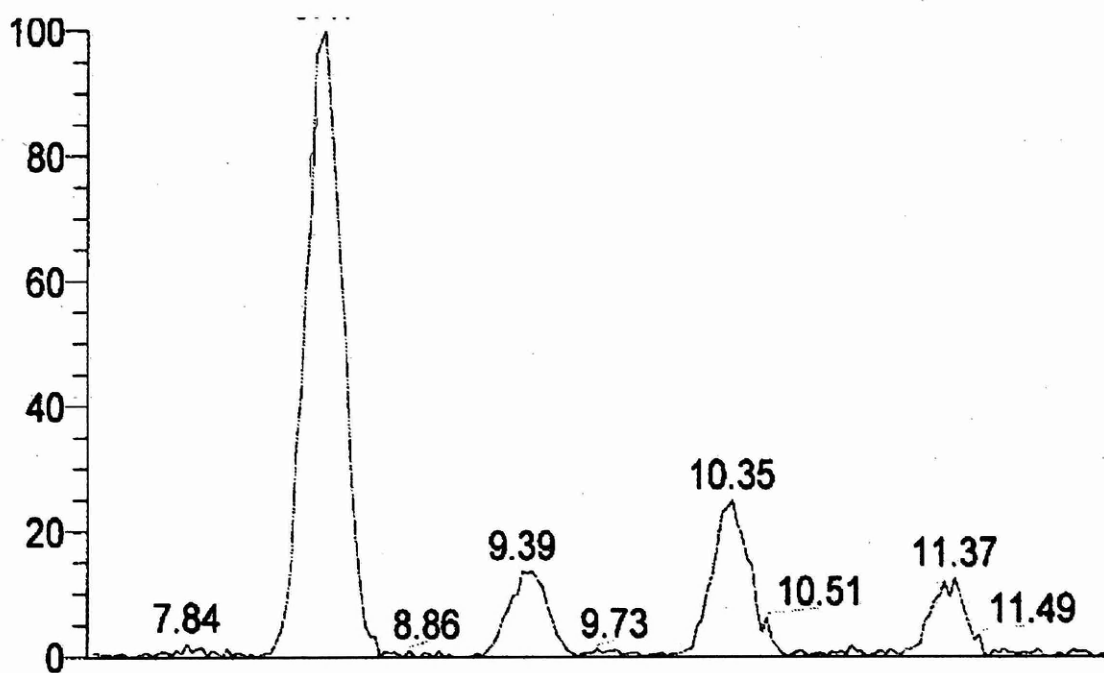


fig 2.4.1: 173 single ion chromatogram of extract p015-5624 run at 1 ml/min on analytical column. (10 μ l injected. Grad=60%-90% acetonitrile in water over 30 min)

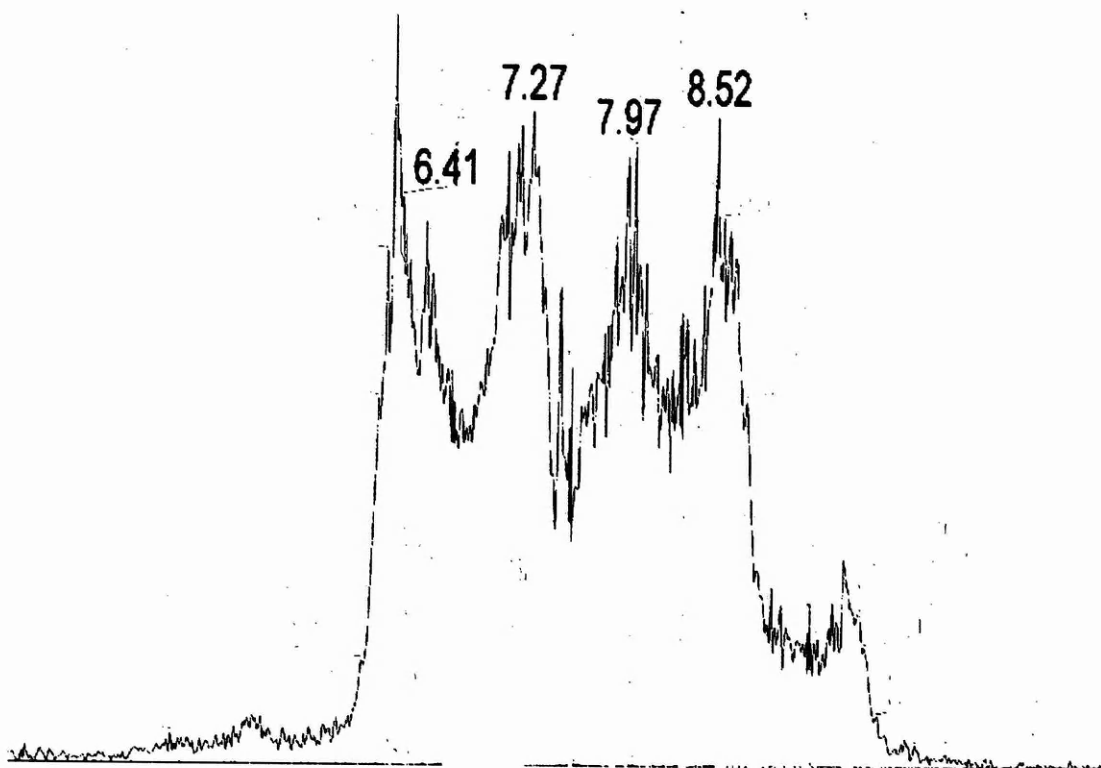


fig 2.4.2: 173 single ion chromatogram of extract of construct p015-5624 run at 5 ml/min on preparative column. (500 μ l injected. Grad=60%-90% acetonitrile in water over 60 min)

It can be seen from figs 2.4.1 and 2.4.2, that the chromatographic separation achieved from the attempts to scale up from analytical (fig 2.4.1) to preparative quantities (fig 2.4.2) using an analytical instrument are quite unsatisfactory. Another problem that arose was the issue of timing. The peaks were collected at the splitter by observing the computer screen and collecting the appropriate peak when it was seen, however this relies on the time taken for the solute to reach the mass spectrometer source and the test tube being exactly the same. As this is often not the case, peaks are often missed. Due to poor chromatography and the timing problem, very few compounds were isolated successfully.

Real advances were made when a Waters platform prep LC system became available. This gave high flow rates for preparative work and had fraction collection capabilities. Initially, with this system, no lactones could be detected under these conditions probably because of the relatively high minimum detection limits of electrospray LC/MS of lactones when using the LCQ. A synthetic standard solution of a triketide propionate lactone was infused into the source via a syringe pump and the instrument tuned to this signal. Once tuning had taken place, the analyte lactones were easily detectable. Scaling up to preparative levels was also much better and preparative runs were usually comparable to the corresponding analytical runs. It was found that the optimal gradient for the separations of these lactones was 60%-90% acetonitrile in water over 30 min. These conditions gave good base line separation allowing pure peaks to be isolated effectively.

Altogether two peaks, (1) and (3) (see fig 2.3.2) were successfully isolated from the construct p015-5624. The first peak isolated proved to have a ^1H NMR spectrum identical to that of a synthetic standard produced earlier,³⁷ which had the exact stereochemistry to be expected from the operation of the DEBS 3 cassette, namely uduu. The ^1H NMR for the peak (3), from here on referred to as the unknown, is discussed in section 2.3

Accurate mass by electrospray ICR-MS:

Peak (1)

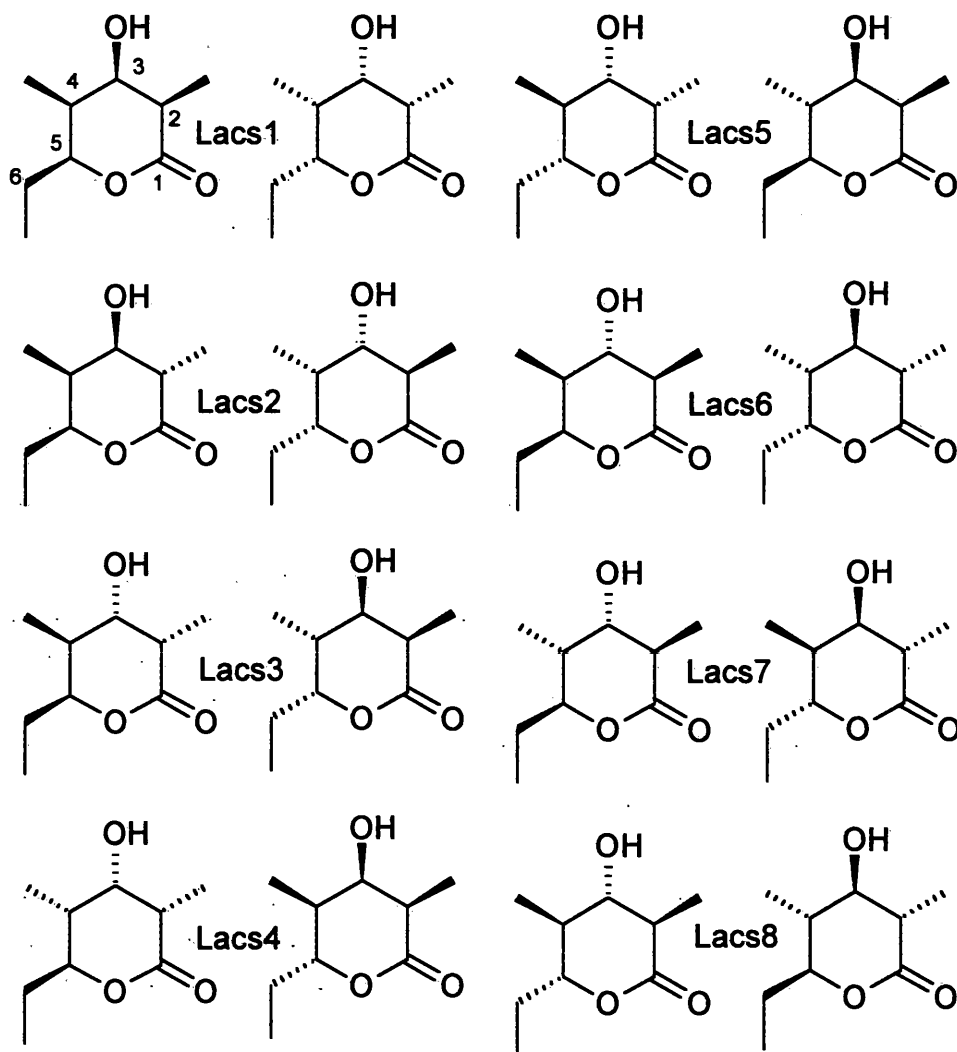
(Found: Na-M^+ , 195.09835. $\text{C}_9\text{H}_{16}\text{O}_3\text{Na}$ requires 195.09916).

Peak (3)

(Found : Na-M^+ , 195.09837. $\text{C}_9\text{H}_{16}\text{O}_3\text{Na}$ requires 195.09916).

2.5 Interpretation of NMR Spectra^{35,36}

As it is not possible to differentiate between pairs of enantiomers of the various triketide lactones by ¹H NMR spectroscopy in non-chiral solvents, the pairs have been grouped together and each pair has been given a code from Lacs1 to Lacs8 as follows:



The first lactone shows the numbering convention used for NMR assignment.

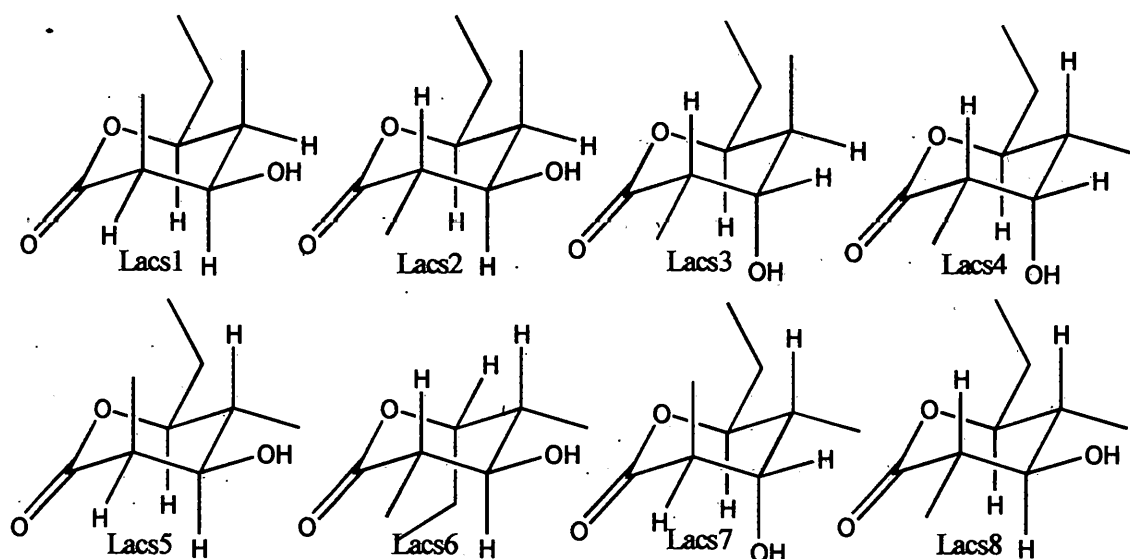
The pure unknown lactone, isolated by preparative HPLC, gave the following 800 MHz NMR spectrum:

δ_H (800 MHz, $CDCl_3$) 4.70 [ddd, 1H, $J=2.9, 5.7, 8.5$ Hz, C(5)-H], 3.6 [d, 1H, $J=2.86$ Hz, C(3)-H], 2.67 [dq, 1H, $J=3.6, 7.2$ Hz, C(2)-H], 2.10 [m, 1H, C(4)-H], 1.81 [m, 1H, C(6)-Ha], 1.55 [m, 1H, C(6)-Hb], 1.36 [d, 3H $J=7.24$ Hz, C(2)- CH_3], 1.03 [t, 3H $J=7.5$ Hz, C(7)- H_3], 1.01 [d, 3H, $J=7.3$ Hz, C(4)- CH_3].

The hydrogen at C(2) gave a signal at 2.67 which consists of a doublet ($J=3.6$) of quartets ($J=7.3$). This shows that the coupling between the C(2)-H and the adjacent ring hydrogen at C(3) is 3.6. That means that the dihedral angle between C(2)-H and C(3)-H must be small (either equatorial-equatorial or axial-equatorial but not axial-axial). Computer molecular modeling using a silicon graphics O2 computer and the molecular modeling program Macromodel 5.5 (Schrodinger Inc. N.J. USA) was performed to calculate the dihedral angles between the adjacent protons on the various lactones. The results are given in table 2.5.1. From these, it was possible to eliminate Lacs2, Lacs6 and Lacs8. The basis for this was given by Karplus³⁵ and assumes that for two adjacent protons with a large dihedral angle i.e. approaching 180° for an axial-axial interaction, the coupling constant between them will be ca. 7.3Hz. For a small axial-equatorial or equatorial-equatorial angle the coupling constant is about 3.6Hz.

	Lac 1	Lac 2	Lac 3	Lac 4	Lac 5	Lac 6	Lac 7	Lac 8
Dihedral angle between hydrogens at C2 and C3	-60.2	144.1	52.1	60.9	68.1	-150.2	-69.4	-164.9
Dihedral angle between hydrogens at C3 and C4	64.1	40.5	60.2	-65.4	95.5	92.6	-64.6	-176.3
Dihedral angle between hydrogens at C4 and C5	-40.6	-59.6	50.5	168.1	-153.9	62.6	169.1	-177.3

Table 2.5.1 Computer modelling prediction of the dihedral angles in the possible stereoisomers of propionate triketide lactones



The above structures give a clearer indication of the dihedrals between adjacent protons. Only one of each pair of enantiomers is shown as the angles are identical within each pair.

The ring hydrogen at C(4) gave a multiplet signal at 2.10. A second NMR experiment was then carried out where the signal was homodecoupled at 0.98 (the peak which corresponds to the C(4)-Me). The signal at 2.10 then consisted of a triplet with coupling constant of 3.5 Hz. This would indicate that the coupling constants between the ring hydrogen at C(4) and the adjacent ring hydrogens at C(3) and at C(5) are the same value, (since the splitting from the methyl group hydrogens at C(4) was effectively eliminated) showing that both dihedral angles are small. The only stereoisomers where all three ring hydrogen dihedral angles are small are Lacs1 and Lacs3. One of the enantiomers of Lacs3 has been synthesised previously³⁷ and the ¹H NMR spectrum of the unknown triketide lactone was found to be identical to that of the published data. Of the two possible enantiomers only the uudd (lacs 3) isomer is really plausible in terms of the operation of the triketide synthase. This is because with the enantiomer dduu, the change from the expected isomer uduu occurs in module 6 whereas with the enantiomer uudd the change occurs in module 5. This is more likely as this is where the change has been made. There is no reason to suspect that the change in KS₁ should affect module 6 but not affect module 5.

2.6 Implications of results

There were two main propionate lactones produced. The first proved to be the stereoisomer (uduu) formed by the normal operations of modules 5 and 6 which had been isolated earlier in studies on triketide formation by an unmodified version of DEBS3³⁷. This result confirms that KS₁ can deliver the unepimerised (2R)-keto ester to its partner KR domain in the same module. The second major isomer which was characterised in chapter 2.9, was identical by proton NMR spectroscopy to another stereoisomer (uudd) isolated in earlier work.³⁷ This differed from the normal product at the three chiral centres C-3, 4, 5. The most significant point of difference in the context of the current investigation was the stereochemistry at C4 which corresponds to the (2S)-isomer produced by epimerisation of the keto ester at the methyl branching site of the diketide formed in module 5. Surprisingly, in accepting this opposite epimeric form of the keto ester, KR₅ reduces the keto group not in its usual sense by hydride addition to the Re face to give the (3S)-3-hydroxy product but in the opposite sense to give the (3R)-product (fig 2.6.1).

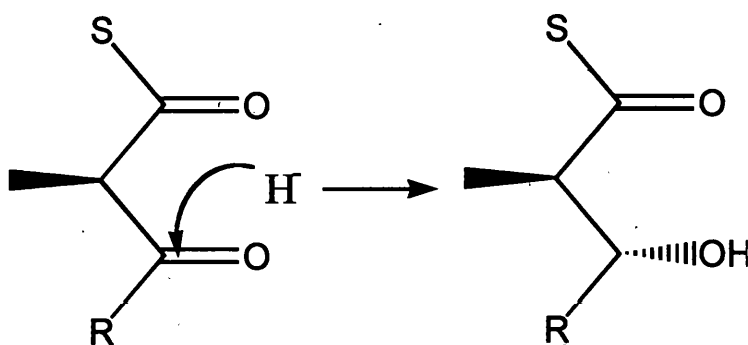


fig 2.6.1 hydride addition occurs at Si face

The unexpected reduction behaviour then continues in module 6. The condensation proceeds as expected to give the non-epimerised (2R)-keto ester but KR₆ then reduces this by hydride addition not in its normal mode to the Re face but to the opposite Si face. Consequently, the stereochemical relationship between C2 and C3 in the second chain

extension unit is *anti* rather than *syn*. Remarkably, this altered stereospecificity in the reduction step is triggered not by alteration of the configuration at the branching methyl centre generated in the current chain extension reaction, but by anomalous chiral centres generated in the previous chain extension module.

The most significant outcome of these experiments, however, is the demonstration that module 5 can be turned from a non-epimerising environment to one capable of epimerisation merely by transfer of KS₁ to replace KS₅. We believe that the two loading domains can be excluded from this role because of the earlier work by Katz³⁸ who showed that removal of these domains from the front of module 1 in the otherwise intact erythromycin PKS did not destroy the epimerising activity of module 1. Our results clearly implicate KS₁ as the source of epimerising activity, but it is important to stress that it remains to be proven that it produces this change by acting as an active catalyst of the epimerisation process.

The results presented here demonstrate that epimerisation activity can be transferred from one module to another by genetic engineering but, depending on the character of the receptor module, the outcome may be both surprising and unexpected.

2.7 The need for synthetic standards

When carrying out any kind of analysis, probably the single most useful thing to have available is a standard. It makes both qualitative and quantitative analysis much easier by transforming it from being almost trial and error to a simple matter of comparison. This standard should be as close in structure to the analyte (or the expected analyte) as possible. In the case of this report, most of the standards used were of the same structure (or expected structure) as the analyte but different stereochemistry. In doing this, certain assumptions were made concerning the nature of the mass spectrometer. It was assumed that both standard and analyte would give the same response factor, that is, a given quantity of one compound will give the same peak area as the same quantity of another compound. Considering that the two compounds (analyte and standard) were so similar and only differed in terms of stereochemistry it was thought that this was a reasonable assumption to make. Triketide lactones are not commercially available so all standards must be either synthesised or isolated from bacterial culture. The second is possible only if the compound is produced in large enough quantities but the first option is more favourable as it allows exactly the right standard to be made in any quantity.

An attempt was made to synthesise the propionate triketide lactone *uduu*³⁹ (the expected lactone from the DEBS3 construct p015-5624).

Fig 2.7.1 shows the proposed reaction scheme to obtain the appropriate lactone (7). The first step (a) is an aldol reaction. This was carried out under the conditions described in section 2.12.1 in the presence of dibutylboron triflate, to form the dibutyl boron enolate of chiral propionamide (1). The reaction proceeded successfully and the expected anti aldol product (2) was obtained. The second step (b) protected the hydroxyl group using tert-butyldimethylsilyl triflate to give (3) so that the phenylmethyl-2-oxazolidinone could be

removed by reduction with lithium borohydride in the third step (c) to yield the free alcohol (4). This was then oxidised to the aldehyde (5) in the fourth step (d) using the oxidising agent tetrapropylammonium perruthenate (TPAP). When step e, the second aldol reaction was attempted under the same conditions the expected aldol product (6) could not be detected. It was thought that the reaction did not proceed because of the incompatible stereochemistry between the chiral auxiliary (1) and the aldehyde (5) in undergoing an aldol reaction.

The chiral auxiliary (8) of opposite configuration to that used in step e was then employed and the procedure performed under the same conditions as step e (step e'). This time the reaction was successful giving the aldol product (9). This was then cleaved and cyclised using lithium hydroxide and hydrogen peroxide (step f') to yield the propionate triketide lactone duuu (10) corresponding to lacs 2. Full conditions for this synthesis are described in chapter 3.4.

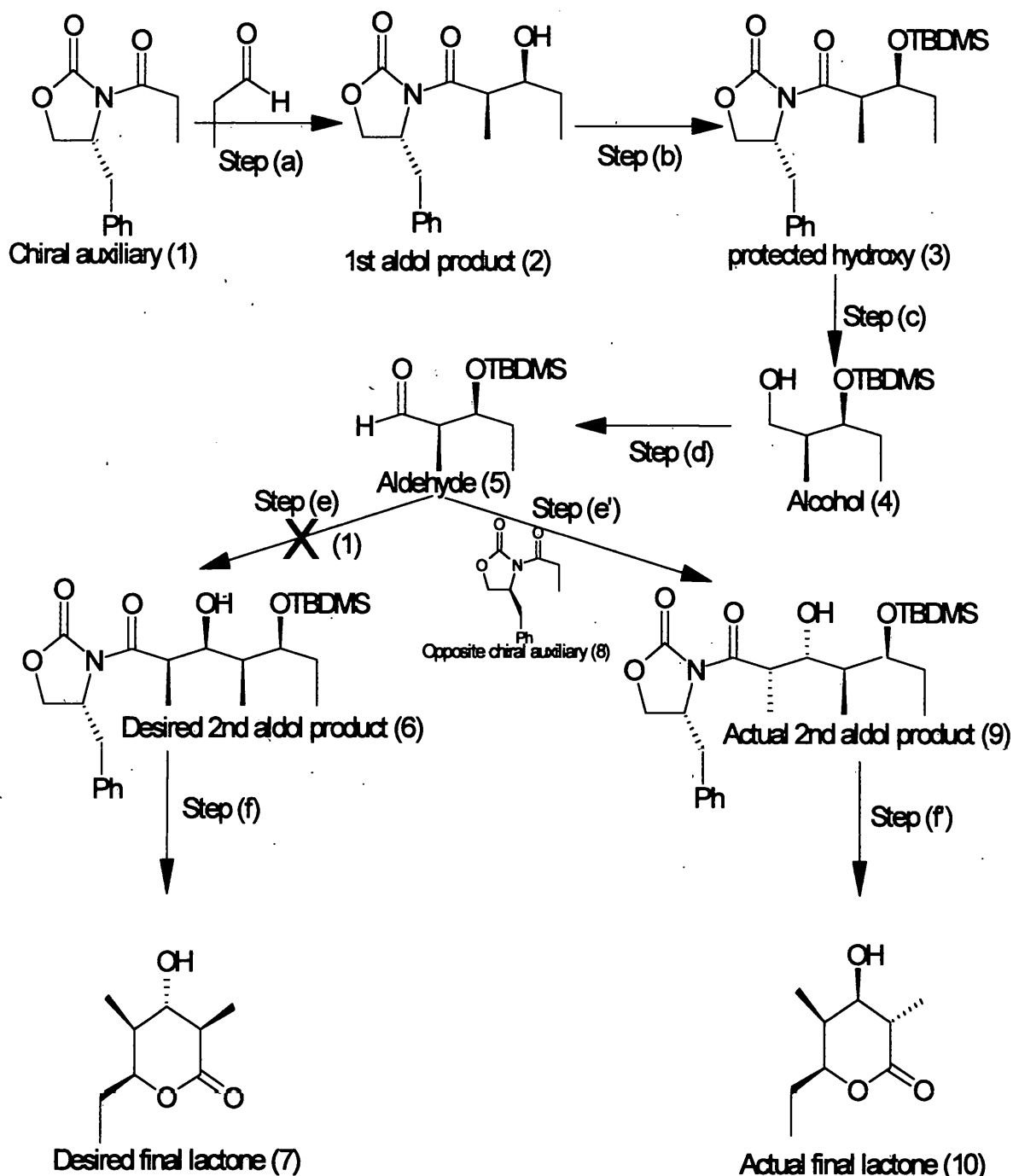


Fig 2.7.1 Steps (a)-(d) show the steps successfully completed. Steps (e) and (f) were unsuccessful however steps (e') and (f') were completed successfully.

Chapter 3

Experimental

3.1 SM3 agar plates

This section describes the process by which the bacterium containing the particular construct was cultivated so that the desired metabolites may be produced in maximum yield

Constituents for preparation of growth media

1. Glucose	8g
2. MD30E	80g
3. Soy Bean Flour	40g
4. Beet Molasses	4.8g
5. K_2HPO_4	0.4g
6. $CaCO_3$	4g
7. Agar	35.2g

The above ingredients were placed into a large beaker containing about one litre of milli-Q water. The mixture was stirred and heated slightly for 20 minutes until everything had dissolved. The mixture was then made up to the 1.6 l mark and stirred. The pH was taken with a pH probe and the pH adjusted accordingly with 1M KOH. The mixture was divided into 200 ml aliquots, which were placed into eight 3 l conical flasks. These were stoppered and heated in an autoclave at 125°C for 1 hour.

When the autoclave cycle was complete and the media cool enough to touch (40°C), thiostrepton solution (200 μ l, 100 μ g/ml) was added. The media was then poured out into eight 500cm² Petri dishes, allowed to cool and covered ready for inoculation.

Seed cultures of the construct were prepared using tryptic soy broth (50ml) containing thiostrepton solution (10 μ , 100 μ g/ml). These were inoculated from storage plates or frozen stocks and incubated and shaken at 30°C, 250 RPM for 3 days. The cells were checked under the microscope and if the cell density was high enough, the plates were inoculated and left to grow at 37°C for approx. 2 weeks when they were extracted.

3.2 Extraction of plates and liquid

This section describes the general procedure employed for the extraction of bacterial culture.

- **Plates**

The first agar plate was sliced into 4cm² squares with a spatula. These were transferred to a Waring blender bowl and 1% formic acid in ethyl acetate (200 ml) was added. This was blended for 10 seconds on low power. The mixture was left to stand for 30 seconds and then blended for a further 10 seconds on low power. It was left to settle again for 30 seconds and the supernatant liquid was decanted off. A further portion of ethyl acetate (200 ml) was added and the plate extracted again. After a third extraction, the extracts were combined. This procedure was repeated for all the eight plates and all the extracts combined. Sodium hydrogen carbonate (200 g) was added and the extract effervesced quite vigorously. The extract was stirred with a magnetic stirrer until it was neutralised (usually about 2 hours) and was then filtered through coarse filter paper using a Buchner funnel. Sodium sulfate (200 g) was added to dry the extract. Once dry, the extract was again filtered through coarse filter paper. The dry, neutral extract was concentrated under reduced pressure ready for analysis and purification.

- **Liquid**

The liquid cultures were removed from the shaker and 3M hydrochloric acid was added to take the pH to about 2. They were then poured into 500ml centrifuge buckets, balanced and spun at 10,000 RPM for 2 hours. After this, the supernatant liquid was carefully decanted off and the cell matter discarded. All the liquid was combined and placed into a large separating funnel. Ethyl acetate (500 ml) was added and the funnel shaken for 1 min. The ethyl acetate layer was separated and the process repeated twice more with fresh portions of ethyl acetate. The ethyl acetate fractions were combined and sodium sulfate (200g) was added. The mixture was then filtered through coarse filter paper and the dry extract concentrated under reduced pressure ready for analysis or purification.

Preliminary purification of crude extracts was performed either by silica gel flash column chromatography or by size exclusion chromatography.

3.3 Isolation of Triketide Lactones from p015-5624 (Ery load-KS₁-DEBS3)

This section describes the method by which this particular batch was grown and harvested.

To yield the pure compounds discussed.

Bacterial seed cultures were plated out onto agar (500cm² x 8) containing thiostrepton (50 µl/ml). These were incubated at 30°C for 14 days until they turned a deep brownish red colour. The plates were then extracted three times into ethyl acetate containing 1% formic acid, using a Waring blender. The extract was neutralised over sodium hydrogen carbonate, left to stir for 2 hours and dried over sodium sulfate. The mixture was then filtered and concentrated under reduced pressure to approx. 10 ml. This crude extract was analysed by GC-MS (temperature gradient 40°C-145°C in 23 mins) on a 30m, 0.25mm ID Rtx-5MS capillary column (Thames Restek UK LTD, Windsor, UK) for the presence of triketide lactones. The extract was then loaded onto a column of silica (14cm diameter x 14cm length). This was eluted with diethyl ether (400ml), 25% ethyl acetate in diethyl ether (400ml), 50% ethyl acetate in diethyl ether (400ml), 75% ethyl acetate in diethyl ether (400ml) and ethyl acetate (400ml). The eluent was collected in 8 x 250ml fractions that were analysed by GC-MS for the presence of triketide lactones. The appropriate fractions were combined, concentrated to dryness under reduced pressure and dissolved in HPLC grade acetonitrile (1.5 ml). To this was added milli-Q water (0.5 ml). The solution was filtered through a 0.2 micron filter, ready for preparative HPLC.

A portion of the semi-pure extract (500µl) was then loaded onto a preparative LC system using a reverse phase Prodigy 5µ 100Å ODS column (25cm x 2.2cm) (Phenomenex, Macclesfield, UK) and eluted using a linear gradient of 5%-30% acetonitrile over 45 min at 20ml/min. The two major peaks corresponding to propionate lactone were collected. The LC separation was repeated with a further three aliquots. The appropriate fractions were combined, concentrated to dryness under reduced pressure and dissolved in CDCl₃ ready for NMR spectroscopic analysis.

3.4 Synthesis of standard

Step (a)

(4R)-3-[(2R, 3S)-3-Hydroxy-2-methylpentanoyl]-4-phenylmethyl-2-oxazolidinone (2)

Dibutylboron triflate (2.6 ml, 12.05mmol, 1.17 eq) and triethylamine (1.648 ml, 11.84mmol, 1.15eq) were added dropwise to a solution of (4R)-3-propionyl-4-phenylmethyl-2-oxazolidinone (2.4g, 10.30mmol, 1eq) in dry dichloromethane (80 ml) at 0°C. This solution was stirred for 1 h, cooled to -78°C and propionaldehyde (0.816 ml, 11.33mmol, 1.1 eq) was added dropwise. The solution was stirred for 1h at -78°C and then for 1.5 h at 0°C. The reaction mixture was quenched by the addition of pH 7 phosphate buffer (16 ml) and methanol (48 ml). A mixture of methanol-30% aqueous hydrogen peroxide (2:1) (43.2 ml) was added dropwise at 0°C and the mixture stirred for 1 h. The volatile solvents were carefully removed under reduced pressure and the remaining aqueous slurry extracted with dichloromethane (3×80 ml). The combined extracts were dried over MgSO₄, filtered and the solvent was removed under reduced pressure to give the crude product. This was purified by flash chromatography eluting with EtOAc : in light petroleum (bp 40-60°C) 50 : 50 to give the aldol adduct (2) (2.2g) 74%

$[\alpha]_D -41.2^\circ$ (c 2.6 in CHCl₃);

ν_{\max} (thin film)/cm⁻¹ 3487 (OH), 1770 (oxazolidione C=O), 1665 (amide C=O);

δ_H (500 MHz; CDCl₃) 7.36-7.17 (5H, m, Ph), 4.69 (1H, m, 4-H), 4.2 (2H, m, 2×5-H), 3.86 (1H, m, 3'-H), 3.79 (1H, dq, $J=2.61, 7.0$ Hz, 2'H), 3.24 (1H, dd, $J=13.4, 3.3$ Hz, PhCHH), 2.84 (1H, d, $J=2.7$ Hz, OH), 2.77 (1H, dd, $J=13.4, 9.5$ Hz, PhCHH), 1.60-1.40 (2H, m, 2×4'-H), 1.25 (3H, d, $J=7.0$ Hz, 2'-CH₃), 0.97 (3H, d, $J=7.4$ Hz, 4'-CH₃);

(Found: M⁺, 291.1474. C₁₀H₂₁NO₄ requires M, 291.1471).

Step (b)

(4R)-3-[(2R,3S)-3-(*tert*-Butyldimethylsilyloxy-2-methylpentanoyl)]-4-phenylmethyl-2-oxazolidinone (3)

To a solution of (4R)-3-[(2R,3S)-3-hydroxy-2-methylpentanoyl]-4-phenylmethyl-2-oxazolidinone (2.204g, 6.81 mmol, 1eq) in dry dichloromethane (59.6 ml) at -78°C were successively added diisopropylethylamine (2.32 ml, 13.63mmol, 2eq) and *tert*-butyldimethylsilyl triflate (1.638 ml, 7.51 mmol, 1.1eq). This solution was stirred until the reaction mixture was seen to be complete by t.l.c. (2h). The volatile materials were then removed under reduced pressure and the crude product purified by flash chromatography (EtOAc : petroleum ether 3:7 v/v) to afford (3) (2.75g, 84%);

δ_H (500 MHz; CDCl₃), 7.36-7.17 (5H, m, Ph), 4.69 (1H, m, 4-H), 4.2 (2H, m, 2×5-H), 4.0 (2H, m, 2'-H, 3'-H), 3.3 (1H, dd, $J=3.2$, 13.3Hz, PhCHH), 2.75 (1H, dd, $J=9.7$, 13.3Hz, PhCHH), 1.60-1.40 (2H, m, 2×4'-H), 1.2 (3H, d, $J=6.7$, 2'-CH₃), 0.9 (12H, m, Si-^tBu +4'-CH₃), 0.11 (3H, s, Si-CH₃), 0.09 (3H, s, Si-CH₃)

Step (c)

(2R, 3S)-3-*tert*-Butyldimethylsilyloxy- 2-methylpentanol (4)

To a solution of (4R)-3-[(2R, 3S)-3-*tert*-butyldimethylsilyloxy-2-methylpentanoyl]-4-phenylmethyl-2-oxazolidinone (2.75g, 6.8mmol, 1 eq) in diethyl ether (96ml) at 0°C was added water (128μl, 7.11mmol, 1.1 eq) and lithium borohydride (3.64 ml of 2M solution in THF, 7.2mmol, 1.1 eq). This solution was warmed to room temperature and stirred for one hour, quenched with 1M sodium hydroxide (64 ml), and stirred for 10 min, then extracted with diethyl ether (3× 65 ml). The organic layers were combined, washed with brine (65

ml), dried over MgSO_4 and concentrated to dryness under reduced pressure. The crude product was purified by flash silica chromatography (EtOAc : petroleum ether 1:4v/v) to afford **(4)** (116mg, 7.4%);

ν_{max} (thin film)/ cm^{-1} 3365 (OH)

Step (d)

(2R, 3S)-3-tert-Butyldimethylsilyloxy-2-methylpentanal (5)

Solid tetrapropylammonium perruthenate (TPAP) (8.78g) was added to a solution of (2R, 3S)-3-tert-butyldimethylsilyloxy-2-methylpentanol (116mg, 0.5mmol 1eq), 4-methylmorpholine N-oxide (87.85 mg) and powdered molecular sieves (325 mg) in dichloromethane (1 ml) at room temperature and under argon. This was stirred for 1h 20min and the crude aldehyde was purified by flash silica chromatography (EtOAc : petroleum ether 1:9v/v) to afford **(5)** (0.065g, 56.5%).

$[\alpha]_{\text{D}} -21.2^\circ$ (c 1.10 in CHCl_3);

ν_{max} (thin film)/ cm^{-1} 1727 (C=O);

δ_{H} (500 MHz; CDCl_3), 9.67 (1H, d, $J=1.0\text{Hz}$, CHO), 4.02 (1H, td, $J=6.6, 3.6\text{Hz}$, 3-H), 2.45 (1H, qdd, $J=6.9, 3.6, 1.0\text{Hz}$, 2-H), 1.59-1.45 (2H, m, 2x4-H), 1.04 (3H, d, $J=6.9\text{Hz}$, 2- CH_3), 0.87 (3H, t, $J=7.5\text{Hz}$, 5- CH_3), 0.84 (9H, s, Si- ^tBu), 0.06 (3H, s, Si- CH_3), 0.02 (3H, s, Si- CH_3);

(Found: $[\text{M}-\text{H}]^+$, 231.1707. $\text{C}_{12}\text{H}_{26}\text{O}_2\text{Si}$ requires M, 231.1703).

Step (e')

(4R)-3-[(2R, 3S)-3-Hydroxy-2-methylpentanoyl]-4-phenylmethyl-2-oxazolidinone (9)

Dibutylboron triflate (0.29 ml, 1.34mmol, 1.17eq) and triethylamine (0.19 ml, 1.30mmol, 1.15eq) were added dropwise, successively to a solution of (4S)-3-propionyl-4-phenylmethyl-2-oxazolidinone (**8**) (0.27 mg, 1.17 mmol, 1 eq) in dry dichloromethane (7 ml) at 0°C. The solution was stirred for 45 min then cooled to -78°C and (2R, 3S)-3-*tert*-butyldimethylsilyloxy-2-methylpentanal (0.0295 g, 1.28mmol, 1.1 eq) in dry dichloromethane (3 ml) was added dropwise. The solution was stirred for 30 min at -78°C, 75 min at 0°C and then 30 min at room temperature. The reaction mixture was then quenched by the addition of pH 7 phosphate buffer (4 ml) and methanol (12 ml). A mixture of methanol-30% aqueous hydrogen peroxide (2:1) (12 ml) was added dropwise at 0°C and the cloudy mixture stirred for 1 h. The volatile solvents were removed under reduced pressure and the aqueous slurry extracted with dichloromethane (3×20 ml). The extracts were combined, washed with brine (40 ml), dried over MgSO₄ and the solvent was removed under reduced pressure. The crude product was purified by flash silica chromatography (EtOAc : petroleum ether 15:85 v/v) to afford (**9**).

ν_{\max} (thin film)/cm⁻¹ 3475 (OH). 1775 (Oxazolidione C=O), 1700 (amide C=O);

δ_{H} (500 MHz; CDCl₃), 7.34-7.20 (5H, m, Ph), 4.69 (1H, m, 4-H), 4.22-4.08 (2H, m, 2×5-H), 4.00 (1H, dd, $J=9.8, 2.0, 2.0\text{Hz}$, 3'-H), 3.84 (1H, dq, $J=6.9, 2.0\text{Hz}$, 2-H), 3.76 (1H, td, $J=7.3, 2.5\text{Hz}$, 5'-H), 3.34 (1H, dd, $J=13.3, 3.1\text{Hz}$, PhCHH), 2.75 (1H, dd, $J=13.3, 9.8\text{Hz}$, PhCHH), 1.82 (1H, dqd, $J=9.8, 7.1, 2.5\text{Hz}$, 4'-H), 1.54 (2H, d, $J=7.3\text{Hz}$, 2×6'-H), 1.21 (3H, d, $J=6.9\text{Hz}$, 2'-CH₃), 0.89 (3H, t, $J=7.3\text{Hz}$, 7'-CH₃), 0.88 (9H, s, Si-^{*t*}Bu), 0.84 (3H, d, $J=7.1\text{Hz}$, 4'-CH₃), 0.11 (3H, s, Si-CH₃), 0.07 (3H, s, Si-CH₃)

(Found: M^+ , 463.2760. C₂₅H₄₁NO₅Si requires M , 463.2754).

Step (f)

(2S, 3R, 4R, 5S)-3,5-Dihydroxy-2,4-dimethyl-heptanoic acid- δ -lactone (10)

Aqueous hydrogen peroxide (27 wt% in H₂O 100 vol, 0.419 ml, 3.3 mmol, 4.5 eq) and lithium hydroxide (69.8 mg, 7.7 mmol, 2.25 eq) were added successively to a stirred solution of (4S)-3-[(2S, 3R, 4S, 5S)-5-*tert*-butyldimethylsilyloxy-2,4-dimethyl-3-hydroxyheptanyl]-4-phenylmethyl-2-oxazolidinone (344 mg, 0.74mmol, 1 eq) in a mixture of tetrahydrofuran : water (3:1v/v) (18.6 ml) at 0°C. This was stirred for 30 min at room temperature, 75 min at 40°C and cooled to 0°C. It was then quenched with 1.5 M aqueous sodium sulfite solution (2.9 ml, 4.36 mmol, 6 eq). The volatile solvents were removed under vacuum and the aqueous layer acidified to pH 1 with 1M hydrochloric acid and extracted into dichloromethane (3x50 ml). The extracts were combined and concentrated under reduced pressure. 1M hydrochloric acid (23.3 ml) and tetrahydrofuran (4.65 ml) were added to the oil and the mixture stirred at room temperature for 30 min and 40°C for 80 min. The volatile solvents were removed under reduced pressure and the aqueous slurry extracted with EtOAc (3x50 ml). The extract was washed with brine (70 ml), dried over MgSO₄ and purified by flash silica chromatography (diethyl ether) to give the lactone (10) as a white crystalline solid (0.101g, 79.4%);

ν_{\max} (Nujol mull)/cm⁻¹ 3450 (OH), 1715 (C=O)

δ_{H} (500 MHz, CDCl₃), 4.12(1H, td, $J=6.3$, $J=2.3$ Hz, 5-H), 3.81(1H, dd, $J=10.3$, 4.3Hz, 3-H), 2.45(1H, dq, $J=10.3$, 7.1Hz, 2-H), 2.15(1H, m, 4-H), 1.81(1H, m, 1 \times 6-H), 1.58(1H, m, 1 \times 6-H), 1.38(3H, d, $J=7.1$ Hz, 2-CH₃), 0.97(3H, t, $J=7.5$ Hz, 7-CH₃), 0.93(3H, d, $J=7.1$ Hz, 4-CH₃);

(Found: M^+ , 172.1100. C₉H₁₆O₃ requires M , 172.1099)

4.1 Summary and Further Work

Validation of LCQ and GCQ for quantification of lactones was carried out. This showed that the GCQ calibration curve has good linearity to very low detection limits and may be used to accurately quantify triketide lactones.

Optimisation of HPLC system for isolation of small quantities of lactones showed that the isolation and handling of very small quantities of lactone is possible given the right separation conditions.

The method used most for pre-preparative HPLC cleanup was the silica flash column. This proved quite effective but was by no means ideal. The use of size exclusion chromatography proved very effective in separating out a large proportion of the unwanted material from the biological extract. The main drawback of this technique was that dichloromethane had to be used to swell the beads so a mixed solvent gradient could not be used to draw out peaks. As a result, base-line separation of peaks was not achieved. If this procedure could be optimised it would prove very useful in terms of sample throughput, as the column may be washed and used several times, unlike silica which must be re-packed each time.

The successful isolation and characterisation of two products was achieved. The first expected product showed that the DEBS3 area of the erythromycin PKS acts in a way that is expected, even when truncated. The second unexpected product shows not only that KS₁ is capable of epimerising a chiral centre but that this inversion of stereochemistry can be carried through to modules 5 and 6 where the KR domains accept and reduce them in the opposite stereochemical sense, i.e. that they can demonstrate relaxed specificity. This is an

important finding which must be taken into account when designing new polyketide synthases.

The fact that one of the isolated peaks was present in one growth culture and not in another seemingly identical one would indicate that work needs to be carried out surrounding the nature in which these organisms grow within the media. Optimisation of growth media would seem an obvious area for investigation, as little is known about exactly how varying the relative amounts or types of electrolytes and nutrients would affect the production of metabolites.

There are, of course, many other areas of investigation that would make the isolation and identification of triketide lactones more efficient. These include, among others, automated in-line HPLC-NMR and solid phase extraction.

5.1 References

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